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HISTOCHEMICAL AND CHEMICAL STUDIES OF CALCIFEROL-INDUCED VASCULAR INJURIES

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SEVERAL extensive studies have been published on the histological aspects of vascular and other injuries inducible by calciferol intoxication (Reed, Struck and Steck, 1939 ; Ham and Lewis, 1934 ; Ham and Portuendo, 1933 ; Gillman and Gilbert, 1956*a*, *b* ; Gillman, Hathorn and Penn, 1957 ; Gillman and Hathorn, 1957). However, very little information is available about the histochemical and quantitative chemical changes in various connective tissue components, and especially in arteries, under the above circumstances.

In previous studies from this laboratory we attempted to draw attention particularly to the histological and histochemical changes induced in the connective tissues of several different arteries by acute calciferol intoxication (Gillman *et al.*, 1957 ; Gillman and Hathorn, 1957 ; Gillman, 1957). Special emphasis was laid by us on the similarities between such injuries to blood vessels and the repair thereof, and those previously described for healing cutaneous wounds (Dunphy, Udupa and Edwards, 1956 ; Jackson, 1957). It has been, and still is our contention, backed now by far more data, that at least some forms of vascular sclerosis may be the late sequelae of intra-mural scar formation—such scars developing far more slowly in injured arteries than in cutaneous wounds (Gillman, 1957 ; 1958 ; 1959*a* ; Gillman *et al.*, 1957 ; Gillman and Hathorn, 1957 ; 1959).

While histochemically there appeared to be very little doubt that one of the late end results of acute calciferol intoxication, namely sclerosis, was indeed the outcome of intramural scar formation, it seemed worthwhile to conduct on arteries, chemical studies of the type so successfully performed by others for healing cutaneous wounds (Dunphy *et al.*, 1956 ; Jackson, 1957 ; Grillo, Watts and Gross, 1958).

The object of the present paper is, therefore, to report the results of such a combined histological, histochemical and quantitative chemical study of the genesis of calciferol-induced vascular injuries in the rat studied from the first few days after initiation of such injuries and for a period of up to 5 months thereafter.

MATERIAL AND METHODS

Experimental.—Wistar strain rats weighing between 135–360 g. were used in the various experiments, and calciferol in arachis oil (15 mg. per ml.) was administered orally daily with a standard dropper for 5 consecutive days, as shown in Table I. All rats were weighed at

TABLE.—*Details of Experiments*

Exp. No.	Daily dose of calciferol (units)	Number of rats: Male	Number of rats: Female	Remarks
1 (*a*)	25,000	24	8	Examined histochemically at intervals up to 197 days.
(*b*)	37,000	19	—	
(*c*)	50,000	21	—	
(*d*)	75,000	3	—	
2	25,000 (1st course)	6	10	After 116 days, given 2nd course of calciferol.
	37,500 (2nd course)			Used for histochemical observations.
3	150,000 units per kg. body weight	90	—	10 calciferol-treated and 10 controls killed at 1*, 3*, 5, 7*, 10, 20, 40, 80 and 160 days. Used for chemical determinations.
	Nil (controls—pair-fed)	90	—	

* Serum Ca^{++} and SMP only.

intervals of approximately 1 week, and their clinical condition observed. In experiment 3, male rats weighing 219 $\pm$ 2 g. were used. The control rats were fed a daily quantity of food equal to that consumed by the calciferol-treated rats, until the 24th day of the experiment, when the food consumption of the latter rats equalled the *ad lib* consumption of the controls.

Histological.—The rats were killed by ether anaesthesia, the body cavities opened and the animals fixed *in toto* in 10 per cent neutral formalin. One week later the following tissues were taken and fixed in fresh 10 per cent formalin : Heart, ascending aorta and arch, thoracic aorta (longitudinal and transverse sections), abdominal aorta, kidney, stomach, colon, small bowel, spleen, mesentery, tracheal, bronchial and laryngeal cartilages and any other obviously injured tissues. Serial sections of wax embedded tissues were cut of all specimens and these were mounted on serially numbered slides. Successive slides were routinely stained by the following methods :— Weigert's resorcin-fuchsin and haematoxylin, periodic acid Schiff routine (PAS), von Kossa's method, Mallory's phosphotungstic acid haematoxylin (PAH), and alcoholic toluidine blue. Selected specimens were also recut and serial sections stained by the Rheinhart-Abul Haj ferrocyanide method for mucopolysaccharides, Wilder method for reticulin, a modified Masson method for collagen as well as several other stains including alcian blue and van Gieson's method.

Without knowledge of the nature of the treatment, the degree of injury and particularly the amount of PAS and metachromatic and ferrocyanide positive mucopolysaccharide present, collagen and von Kossa positive material was estimated by one of us (TG) according to a rough quantitative histological estimation, for each of the above mentioned findings.

Chemical.—The rats in experiment 3 were sacrificed by ether anaesthesia, and blood was obtained by cardiac puncture. Freshly excised tissues were freed from connective tissue, rinsed in saline, cut into small pieces, pooled and placed under dry acetone. After 24 hr. the acetone was replaced by fresh and allowed to stand for a further 24 hr. period. The acetone was then replaced with ether-alcohol (1 : 1) and after standing overnight the solvent was removed and the tissues dried to constant weight in a hot air oven at 100°.

Extraction of Carbohydrate and Calcium.—Portions of dry tissue (50 mg.) were heated with 4 ml. of 0·5 N hydrochloric acid for 4 hr. at 100° in sealed tubes. The solutions were then filtered and the volumes adjusted to 10 ml.

Sugar estimations.—Hexoses were determined using anthrone according to the method of Trevelyan and Harrison (1952), the results being expressed as glucose.

Amino sugars.—Hexosamines were determined by a modification of the Elson and Morgan method (1933). The acetylacetone reagent was made by dissolving 1 ml. of redistilled acetylacetone in 25 ml. of 1 M sodium carbonate (A.R.) solution. To 1 ml. of sample (containing 5–50 μg. hexosamine) was added 2 ml. of reagent and the mixture heated for exactly 15 min. in a boiling water bath. After cooling, 6 ml. of diluted PDA reagent was added, this consisting of 1·6 g. pure *p*-dimethylaminobenzaldehyde dissolved in a mixture of 30 ml. pure ethanol and 30 ml. concentrated hydrochloric acid and diluted with 300 ml. pure ethanol. The tube was allowed to stand at room temperature for 10 min. with shaking to remove CO_2 and the colour density of the solution was determined at 530 mμ. in a Zeiss spectrophotometer, model M QII, using 2 cm. cells, each run including a duplicate standard (50 μg. pure glucosamine).

Calcium.—Calcium was determined in 1 ml. samples of the acid extract (diluted with 2 ml. distilled water) by direct titration with disodium ethylene diamine tetracetic acid (·01 N), in the presence of alkali (1 ml. 2·5 N NaOH), using the calcium indicator of Diehl and Ellingboe (1956), screened with phthymolphthalein (Tucker, 1957). Serum calcium was determined by the same method.

Collagen and Elastin.—These were determined according to the method of Neuman and Logan (1950), the two 3 hr. autoclavings being replaced by single 6 hr. periods, and the urea extraction was omitted.

Serum mucoprotein.—This was determined according to the turbidimetric micro method of de la Huerga *et al.* (1956). The turbidity was read at 400 mμ in the Zeiss spectrophotometer. Armour bovine serum albumin (M.P.I.) was used for standardisation.

Apart from the phosphate and serum mucoprotein determinations, all chemical analyses were performed by one of us (RAG).

RESULTS

Clinical

After the second daily dose of calciferol, the treated rats lost their appetites and, by the 4th day of the experiment became listless ; their hair became erect, they sat hunched up in their cages and would not, as do normal rats, respond

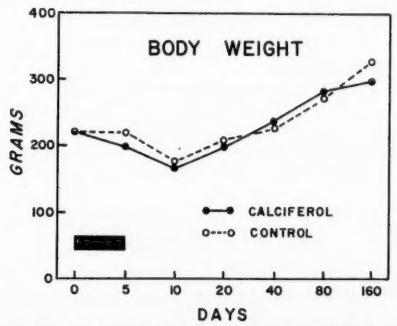


FIG. 1.—Mean body weight of rats in calciferol and control groups during course of experiment. Black bar indicates period over which the 5 daily doses of calciferol were given.

to attempts to attract them by scratching on their cages. Priapism, a recognised sign of Vitamin D intoxication (Reed, Struck and Steck, 1939) was commonly seen as was frequency of micturition and staining of the pelt with urine. Occasional rats died by the 6th day but the most severe toxic reactions and highest death rate occurred after the termination of calciferol dosing *i.e.*, by the 11th to 15th days after the first dose of calciferol.

The growth curves of the animals in Experiment 3 are shown in Fig. 1, from which it can be seen that the calciferol-treated rats lost weight rapidly during the

first 8 to 12 experimental days. They subsequently regained their initial weights by about the 30th day, whereafter they grew at about the same rate as the controls. The fact that their weight curve was almost identical to that of the pair-fed controls suggests that the weight changes were entirely due to the reduced food intake during and immediately after the calciferol administration (Fig. 2).

Macroscopic Findings

Necrosis of the spleen such as that recorded by Gillman and Gilbert (1956*a*, *b*) was encountered in many of our rats that died or were sacrificed by the 15th day. This necrotic change initially seemed to be avascular (Fig. 9) and only later was the heavily congested necrotic splenic tissue regenerated, without scar formation. There were indications that in the survivors, the damaged spleen had regenerated completely by the 25th day. Examples of macroscopic lesions consistently found in other organs are portrayed in Figs. 10 to 14.

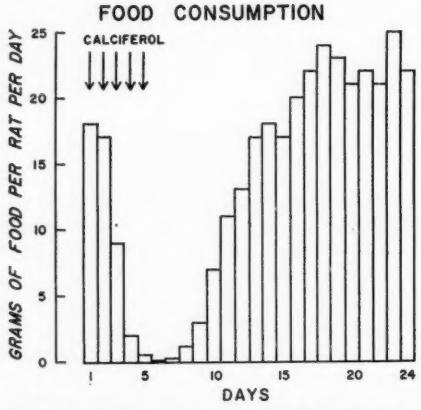


FIG. 2.—Mean daily food consumption per rat in calciferol-treated rats. The control rats, being pair-fed with the experimental rats, consumed the same daily amounts of food.

Microscopic Findings

The histological and histochemical features in rats in Exp. 1 (*b*) and 1 (*c*) were very similar, and the following description is based mainly on these two experiments. Only the lesions in the heart, coronary arteries and aorta will be described in this communication.

Heart.—The heart was severely injured very early in the experiments. The lesions were easily detectable by the basophilia of the damaged myocardium and, as shown in Figs. 15 to 18, such basophilic areas were laden with periodic acid Schiff (PAS) and von Kossa-positive material. These changes in the myocardium resolved rapidly and, by the 9th to 11th day, were replaced by highly cellular tissue diffusely infiltrating the injured areas—especially in the interventricular septum (Fig. 19). This cellularity in turn disappeared, and by the 25th day of the experiment was no longer obvious. Fibrosis of the injured myocardium was not apparent at this stage and only much later were derangements in the reticulin

pattern apparent (Fig. 20). Such late fibrosis, we believe, was not the end result of the initial necrosis but rather perhaps of some subsequent interference with myocardial integrity attendant on the coronary sclerosis and ensuing ischaemia (see below).

The myocardial necrosis, with calcification and carbohydrate accumulation, subsequent cellularity and perhaps regeneration of heart muscle (Gillman and Gilbert, 1956*b*), followed the roughly quantitative pattern outlined for von Kossa and PAS positive material shown in Fig. 3.

Coronary arteries.—It is not intended here to detail the changes in the coronary arteries encountered by us. This has, to a large extent, been done elsewhere

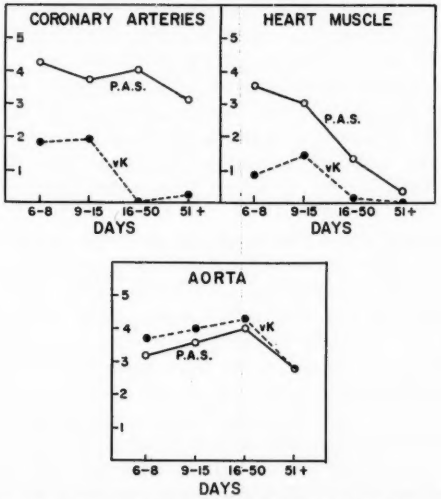


FIG. 3.—Mean arbitrary gradings of intensity of PAS and Von Kossa staining of coronary arteries, heart muscle and aorta, in rats at the indicated stages of Exp. 1.

by others and by us (Ham and Portuendo, 1933 ; Gillman and Gilbert, 1956*a*, *b* ; Gillman *et al.*, 1957 ; Gillman and Hathorn, 1957). However, it is important for the present study to stress the early onset of coronary dilation, injury to the internal elastic membranes, previously described, and the associated early calcification of these structures. The changes associated with repair, and the resulting sclerosis of the coronary arteries, are presented in Figs. 21 to 26. Here are shown the very early dilatation (Fig. 21), " swelling " and intense PAS-positive staining of the internal vascular elastic membranes (VEM) (Figs. 22, 23) and calcification, which resolved rapidly. Although these initial lesions are transient they are soon followed by cellularity of the adventitia and media of the affected arteries, together with both metachromatic and ferrocyanide-positive MPS accumulation (Figs. 22, 23, 24). With larger doses of calciferol, thrombosis of acutely damaged vessels was occasionally observed even in the early stages of the experiment (Fig. 24) ; this was not the case before the 40th day, however, in any of the rats on lower doses of calciferol.

The above early signs of repair were usually followed by very gradual but progressive collagenisation of the media and adventitia associated with intimal fibrin deposits, medial reticulin formation (Figs. 22, 27) and ultimately by marked stenosis with, in many instances, thrombus formation in the much narrowed lumina of the major coronary arteries (Gillman, 1957 ; Gillman and Hathorn, 1957). Marked stenosis, with thrombus formation, was almost invariable in rats in Exp. 2, i.e., those rats receiving 2 courses of calciferol. Almost all the rats treated in this manner, and studied 100 days after the second treatment, had markedly stenosed, partially thrombosed coronary arteries associated with extensive myocardial scarring (Figs. 20 and 28).

Roughly quantitative assessments of the histochemical changes seen in coronary arteries during the development of the initial lesions in Exp. 1 are presented in Fig. 3, in which the amount of early but transient, accumulations of calcium, and the persistence of carbohydrate, are clearly indicated.

Aorta.—Obvious injury, and especially calcification, with increase in perimembranous PAS-positive material, became detectable in the aorta somewhat later than in the heart or coronary arteries in some animals receiving higher doses of calciferol. However, aortic calcification and associated PAS-positive material persisted for very much longer, if not for the remainder of the life of the rats (Fig. 3).

Particularly impressive was the apparent gradient of reactivity in the arterial tree in rats receiving higher doses of calciferol. The heart and coronary vessels reacted earliest, then the ascending aorta and arch, then the thoracic aorta. Only a few days later did the abdominal aorta show the same reactions. Thus, at any particular day in the early stage of the experiments (and especially between days 3 to 10), not all parts of the aorta were equally affected, even though they became so in the experiments. This fact should be borne in mind in interpreting the chemical data which were derived from analysis of aliquots of the entire aorta, i.e., aortic arch down to aortic bifurcation.

Apart from the previously described calcification and necrosis of the tissue immediately surrounding the VEM's (the inner two to three in particular), intimal cellular reactions (Fig. 30) were often prominent (Gillman *et al.*, 1957 ; Gillman and Hathorn, 1957). Mural thrombi were also occasionally seen even in the thoracic aorta. Particularly susceptible to intimal cellularity were the aortic orifices of the coronary arteries (Fig. 31).

Cellularity of the injured aortic media itself was strikingly rare, but at 40 days necrotic and calcified medial tissue, with overlying intimal scars, was commonplace (Figs. 29 and 32). At this stage, the tissue between the fragmented calcified inner 3–4 VEM's was usually laden with metachromatic and ferrocyanide-positive mucopolysaccharide. Cartilage formation too was occasionally encountered even by the 41st day (Fig. 32). However, large plaques of heavily metachromatic cartilage were common in the aortae of rats examined at 60 to 180 days. The persistence of intense aortic calcification with cartilage formation for 100 and more days after the single period of intoxication was also a striking finding in almost all rats.

Chemical Findings

The results of estimations of serum calcium and serum mucoprotein (SMP) in the control and calciferol-treated rats in Exp. 3 are shown in Fig. 4. The mean

dry weights of the aortae are shown in Fig. 5, and the calcium, phosphorus, hexose, hexosamine, collagen and elastin content, expressed as a percentage of

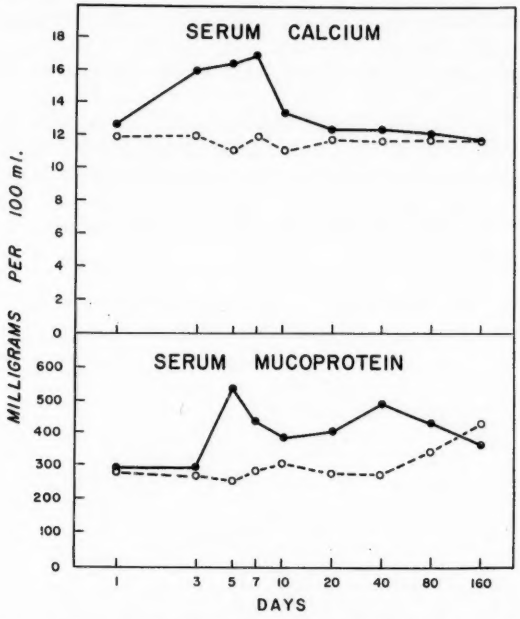


FIG. 4.—Serum calcium and serum mucoprotein in calciferol-treated (solid lines) and control (dotted lines) rats in Exp. 3.

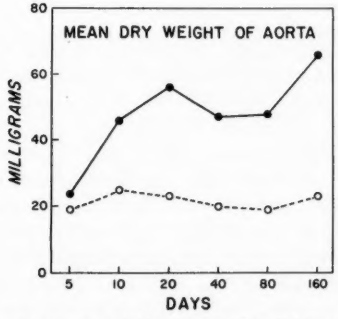


FIG. 5.—Mean dry weights of aorta in calciferol-treated (solid lines) and control (dotted lines) rats in Exp. 3.

the dry fat-free weight, are shown in Figs. 6–7. In Fig. 8 are shown the total amounts of those substances per aorta at different times after the commencement of the experiment.

The data recorded in the accompanying text figures seem to permit the following conclusions :—

The serum calcium levels (Fig. 4) rose soon after calciferol administration had been started and the levels increased until the 7th day. Thereafter, the serum calcium level dropped sharply. By the 10th day the values were only slightly above those of the controls, the latter level being reached by the 20th day of the experiment.

Comparison of Figs. 4 and 6 reveals that maximum aortic calcification occurred after the serum calcium level had fallen, and remained high until the end of the experiment. In this regard our observations confirm those of Trout (1958).

While the serum calcium level had risen sharply by the 3rd day, it should be noted that serum mucoprotein rose only on the 5th day when the serum

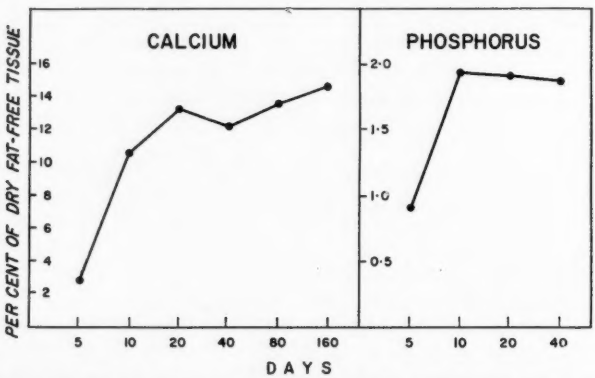


FIG. 6.—Mean calcium and phosphorus content of aorta in calciferol-treated rats in Exp. 3. The values for the control rats were too small to plot, ranging from 0·08 to 0·30 per cent for calcium, and 0·11–0·23 per cent for phosphorus.

calcium was still rising. In contrast to serum calcium, the SMP started to fall by the 5th day, but did not approach control levels until after the 80th day of the experiment, *i.e.*, long after calciferol treatment had stopped and the serum calcium level had dropped to its control level.

Some aspects of these responses in SMP will be briefly discussed below in relation to the similarities between the reactions here encountered and those described by Engel (1952) in parathormone-treated rats. Moreover, in view of the above histological data, it seems worth-while mentioning that the decreasing order of intensity of calcification of the various tissues examined by us, both chemically and histochemically was : —Aorta, lung, gastric muscle, gastric mucosa, heart, skin.

An exceptional amount of calcium accumulated in the aorta, the values in this artery approaching those in bone! (Aorta 13 to 14 per cent calcium ; femur 22 per cent calcium).

As shown in Fig. 6, calcification of the aorta was paralleled by a large increase in its phosphorus content, this heavy deposition of both calcium and phosphate resulting in a two- to three-fold increase in the mean dry weight of the aorta

above that of the controls (Fig. 5). It is noteworthy that the aortic mineralisation was dissociated from the rise in serum calcium level, for the calcification of the aorta continued after the serum calcium had fallen. These large increases in aortic mineral content were not accompanied by any change in the calcium content of the femur.

There was about a two-fold increase in the total " apparent " hexosamine content of the aortae of calciferol-treated rats, this being accompanied by an approximately parallel rise in the total hexose content (Fig. 8). These chemical changes closely parallel the histological gradings on rats in experiment 1 (*b*) (Fig. 3). It is notable too that the increases in aortic hexose and hexosamine

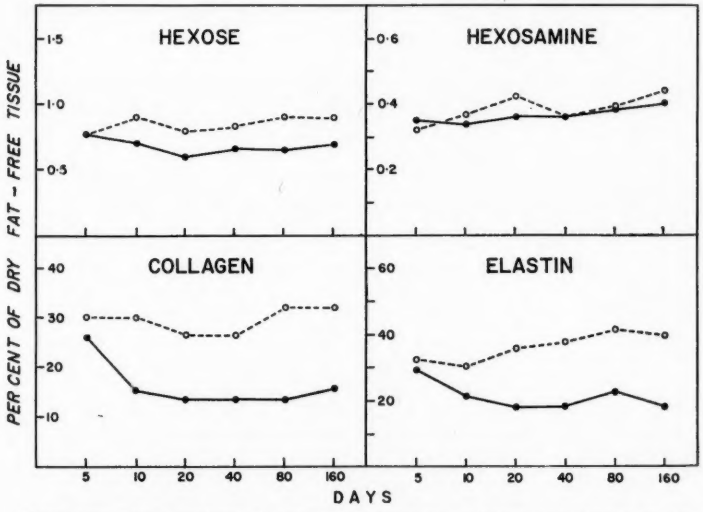


FIG. 7.—Mean percentage content of hexose, hexosamine, collagen and elastin in aortae in calciferol treated (solid lines) and control (dotted lines) rats in Exp. 3.

occurred after the marked initial rise in serum mucoprotein observed between days 5 to 7 of the experiment. However, like the SMP, so too the total aortic hexosamine and hexose values of the calciferol-treated animals once raised, remained high throughout the rest of the experiment. The increases in aortic hexose and hexosamine were, however, of much smaller magnitude than the calcium increase of about a hundred-fold. The only other component which showed a large increase similar to calcium was phosphorus (Fig. 6).

There was a large decrease in both the collagen and elastin percentages of the calcified aorta (Fig. 7). This may be accounted for by the heavy mineralization (*i.e.*, " dilution ") of elastin and collagen by calcium and phosphate.

The total collagen content of the aortae of the experimental animals (Fig. 8) had not risen significantly even by the 80th day of the experiment, a distinct increase over the control level being noted only at the end of the experiment. This chemical finding substantiates the previous report from this laboratory that

EXPLANATION OF PLATES.

FIG. 9.—Spleen of male rat, 7 days after 1st dose of calciferol, Exp. 1*d*. Note extensive areas of necrosis.

FIG. 10.—Stomach of male rat, 7 days. Exp. 1*d*. Note extensive areas of mineralization of stomach wall.

FIG. 11.—Colon of male rat, 11 days, Exp. 1*a*. Note extensive mineralization.

FIG. 12.—Heart of male rat, 7 days, Exp. 1*c*. Note areas of patchy mineralization.

FIG. 13.—Aorta of male rat, 15 days after 2nd dose of calciferol, Exp. 2. Note patchy distribution of mineral in aortic wall.

FIG. 14.—Colon of male rat, 15 days after 2nd dose of calciferol, Exp. 2. Note extensive mineralization of colon, causing intestinal obstruction, which was the cause of death in this rat.

FIG. 15.—Myocardium and commencement of aorta in male rat at 6th day of experiment, i.e., 1 day after last dose of calciferol, Exp. 1*c*. Note heavy mineralization of myocardium and large coronary artery in left of figure. Von Kossa. × 22.

FIG. 16.—Same as above, showing mineralization of larger but not of smaller coronary arteries, and of muscle immediately around these vessels. Von Kossa. × 51.

FIG. 17.—Myocardium of male rat, 7 days, Exp. 1*c*. Note mineralization of myocardium. Von Kossa. × 22.

FIG. 18.—Same field as Fig. 17. Note accumulation of PAS-positive material in regions of calcification shown in Fig. 17. PAS stain. × 22.

FIG. 19.—Interventricular septum of male rat, 10 days, Exp. 1*c*. Note marked cellular infiltration in muscle, with involvement of coronary arteries. No calcium was detected anywhere in this myocardium. Von Kossa. × 51.

FIG. 20.—Myocardium of male rat, Exp. 2, 71 days after second dose of calciferol. Note irregularity in myocardial reticulin in an area of possible old myocardial necrosis. Note also heavy reticulin around small coronary arteries in this field. Wilder stain. × 21.

FIG. 21.—Coronary artery of female rat, 7 days Exp. 2. Note dilatation of this major large coronary artery, and heavy deposition of iron in its wall at the site of calcification. Iron stain. × 48.

FIG. 22.—Coronary artery. Note minimal reticulin in highly cellular media and intima, with considerable increase in adventitial reticulin and collagen. Male rat, 41 days, Exp. 1*b*. Wilder stain. × 224.

FIG. 23.—Medium sized coronary artery from male rat, 43 days, Exp. 1*c*. Note marked thickening and PAS-positive staining of internal vascular elastic membrane, the intact endothelium, the cellular media and adventitia, which contain considerable amounts of PAS-positive material. PAS stain. × 224.

FIG. 24.—Heart of male rat, 18 days, Exp. 1*c*. Advanced stage of coronary lesion, ? early thrombosis. The coronary artery is near the bifurcation of the vessel. PAS stain. × 224.

FIG. 25.—Heart of male rat, Exp. 1*b*. Two coronary vessels with markedly thickened media and eccentric lumina. Tol. blue stain. × 66.

FIG. 26.—Heart of male rat, 41 days, Exp. 1*b*. Coronary artery to show distribution of PAS-positive material, this being in situation of a remnant of the internal vascular elastic membrane. PAS stain. × 224.

FIG. 27.—Heart of male rat, 118 days, Exp. 1*c*. Note marked increase in reticulin of media in the small vessel near the apex, which shows almost complete thrombosis. Wilder stain. × 224.

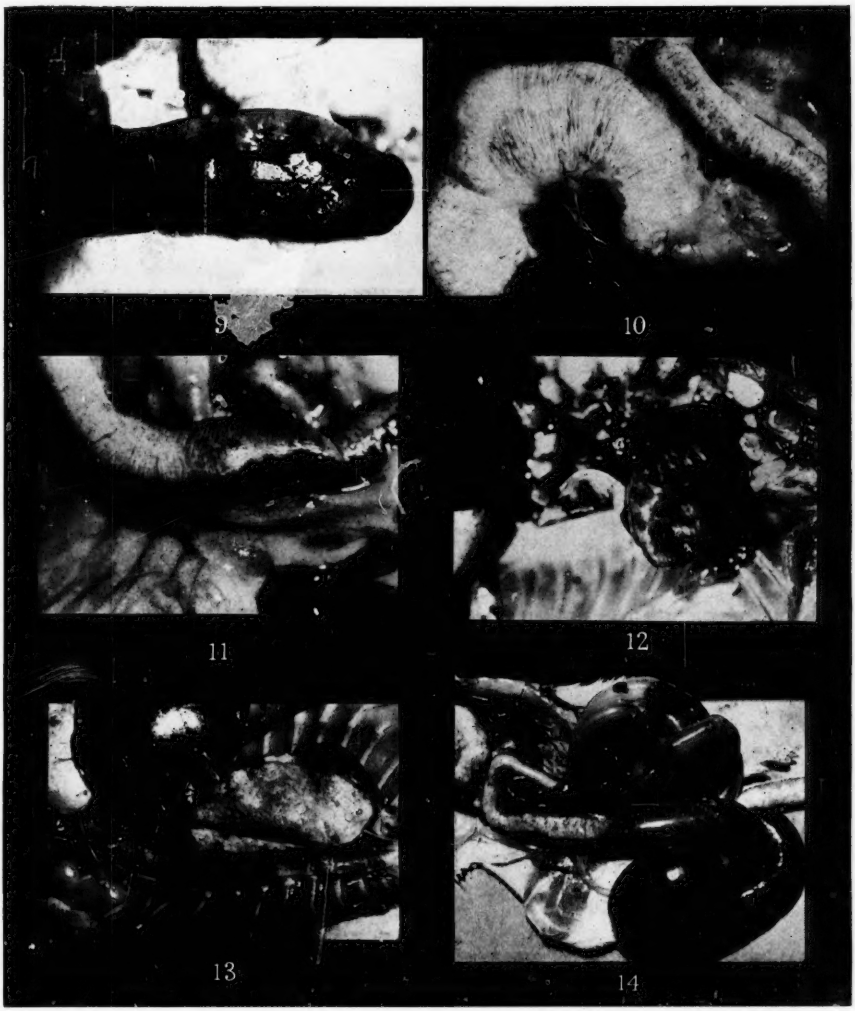
FIG. 28.—Coronary artery of male rat, Exp. 2, 71 days after second dose of calciferol. Note almost complete thrombosis, and heavy PAS-positive accumulation in wall. This reaction was very localized and not detectable in many later serial sections. PAS stain. × 120.

FIG. 29.—Aorta of male rat, 41 days, Exp. 1*b*. Note heavy calcification in area of medial damage and intimal scar. There was no calcification visible anywhere in coronary arteries or in the myocardium. Von Kossa. × 50.

FIG. 30.—Commencement of aorta of male rat, 41 days, Exp. 1*b*. Note thinning of aortic wall and marked cellular reaction in thick intimal scar with extensive thickening of aortic wall over necrotic calcified media. PAH stain. × 50.

FIG. 31.—Origin of main coronary artery of male rat, 10 days, Exp. 1*b*. Note heavy cellular infiltration, moderate endothelial lesion neighbouring mast cells; moderate metachromasia of aortic media and adventitia of coronary artery. Note also thickening of elastic membranes. Tol. blue stain. × 50.

FIG. 32.—Ascending aorta of male rat, 41 days, Exp. 1*b*. Note fragmentation of necrotic medial, PAS-coated elastic membranes; and minimal cellularity of this area of medial necrosis with early cartilage formation, overlying the intimal scar and PAS stain. × 40.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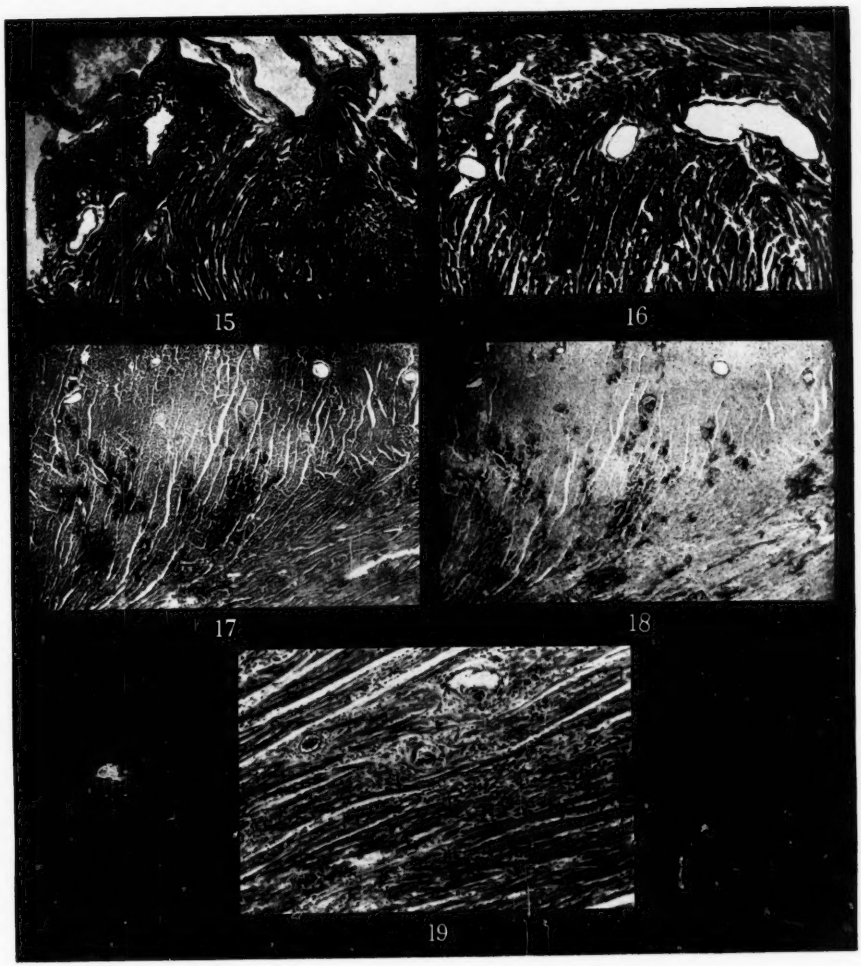

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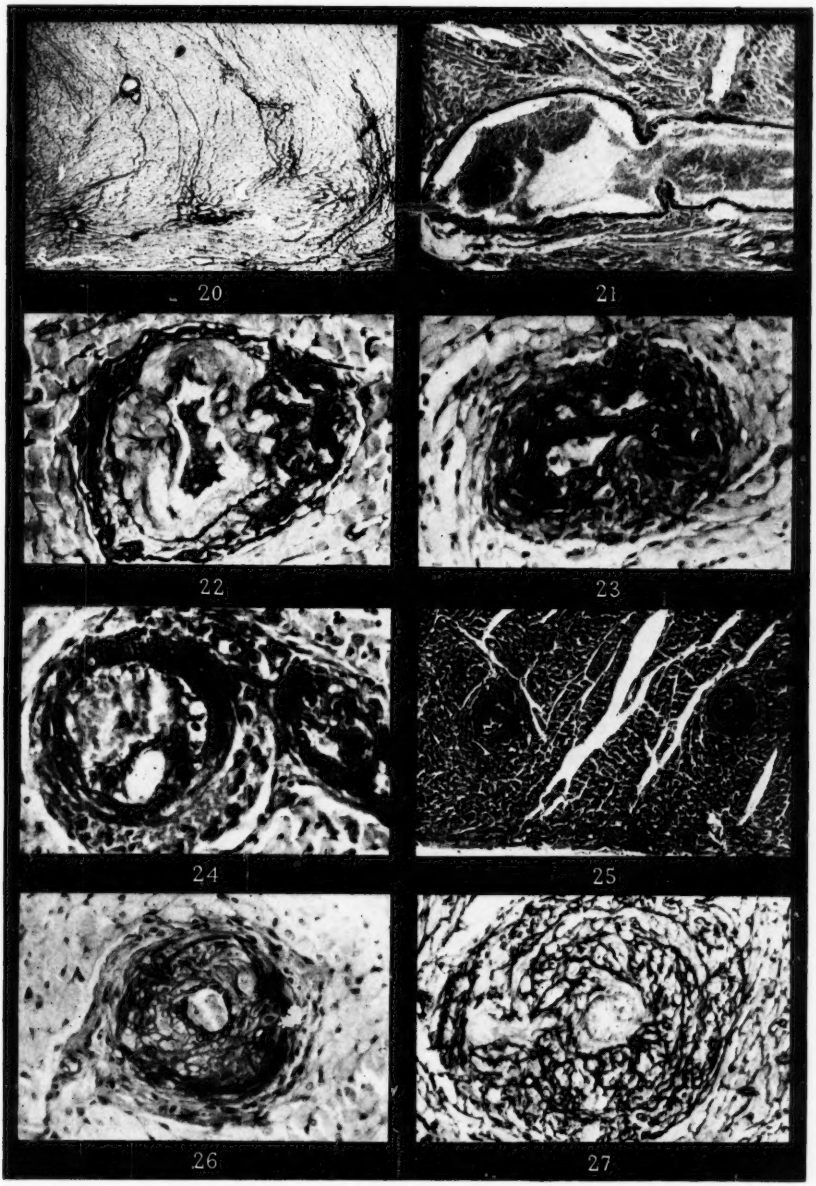

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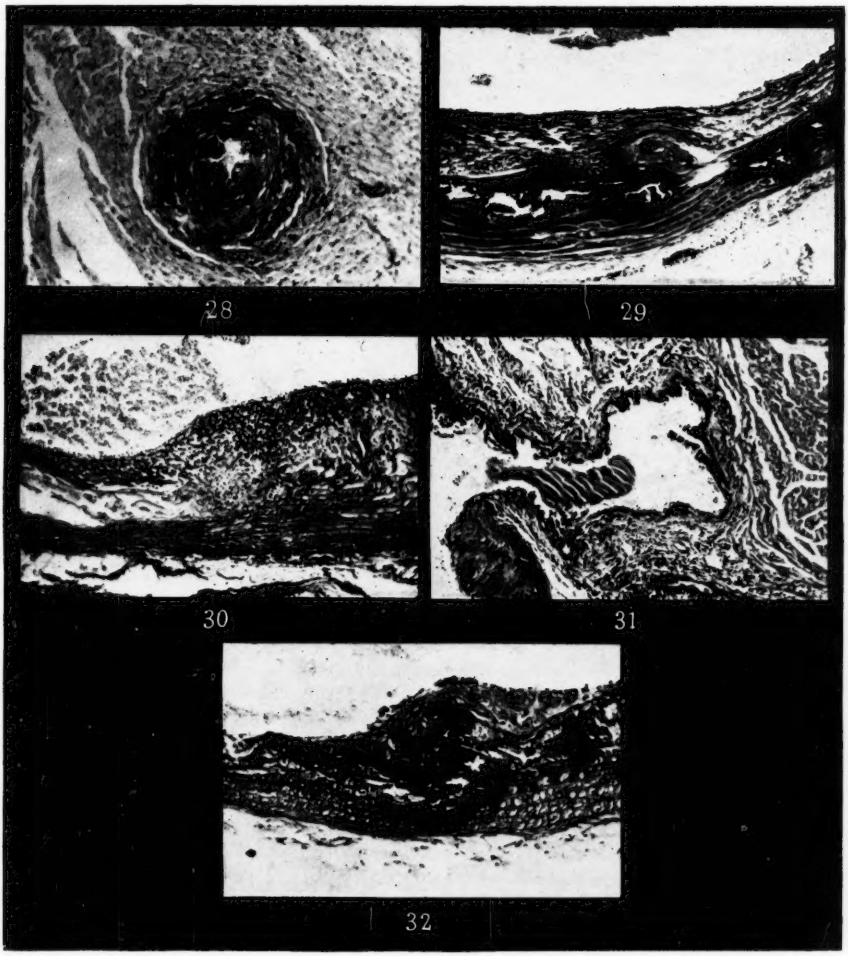

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collagenisation of severely injured vessels takes place far more slowly than is the case in healing cutaneous injuries (Gillman and Hathorn, 1957).

Among the most puzzling of our chemical findings was the change in the total " apparent " elastin (Fig. 8). It will be noted that, compared with the controls, the percentage of " apparent " elastin of the experimental group seemed to decrease in the same way as did the percentage of aortic collagen (Fig. 7). However, as indicated for collagen, this seeming decrease is probably attributable to " dilution " of the connective tissue component by the mineralisation of the aorta. The total " apparent " elastin content, however, was somewhat elevated in the calciferol series during the whole of the experiment (Fig. 16).

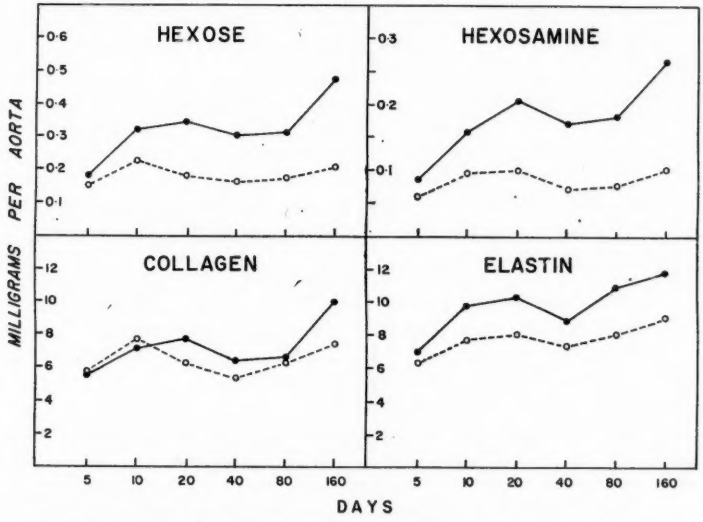

FIG. 8.—Absolute content of hexose, hexosamine, collagen and elastin in aortae in calciferol treated (solid lines) and control (dotted lines) rats in Exp. 3.

Histologically, there was no detectable increase in either elastic or " pseudo-elastic " fibres (the latter defined elsewhere—Gillman *et al.*, 1955). In fact, fractures and fragmentation of the original elastic membranes, consequent on perimembranous mineralization, were common. Such fractures were later followed by marked localised fibrotic reactions, and as already recorded elsewhere, no obvious regeneration of the vascular elastic membranes was noted at any time, even 300 or more days after a single period of calciferol intoxication (Gillman *et al.*, 1957 ; Gillman, 1957 ; 1958 ; 1959*a*). In view of the absence of any histologically demonstrable increase in elastic fibres, we considered the possibility that the apparent increase in chemically estimated elastin may have been partly attributable to the Weigert- and orcein-positive material easily demonstrable within the cartilage plaques which developed in the aortic media. Our initial studies of rat costal cartilages have revealed a small amount of hydroxyproline which is resistant to autoclaving and which may account for the apparent increase in chemically determined elastin.

DISCUSSION

The primary aims of the present combined histological, histochemical and quantitative chemical analyses of the changes in the heart, coronary arteries and the aortae of calciferol-intoxicated rats were to determine :—

(*a*) Whether the microscopic findings were indeed providing a reliable indication of the chemically determinable changes, particularly in the aorta. If the roughly quantitative microscopic assessments of the changes in the aortae are trustworthy, then we assume that such data will be acceptable for other tissues. The comparative chemical and histochemical findings in other tissues, will, however, be discussed and assessed by us in future publications.

(*b*) Whether the sequence of events in severely injured vessels, as delineated microscopically (Gillman *et al.*, 1957 ; Gillman and Hathorn, 1957 ; 1958) could be confirmed by quantitative chemical analyses.

We consider that the data here provided strongly indicate that the previously described microscopically determined changes, particularly in the aorta, are fully supported and somewhat amplified by the present chemical findings.

Perhaps of special interest, in the light of recent work, are our observations on the serum mucoprotein changes in our calciferol-intoxicated rats. For Faber (1949) reported a progressive increase in chemically estimated human aortic mucopolysaccharides, with ageing, and especially in association with cholesterosis. This finding conforms with the histochemical observations of Moon and Rinehart (1952), Taylor (1953) and Moon (1957). More recently, Noble *et al.*, (1957) reported increases in aortic hexosamine with ageing. In conformity with the observations of the histologists quoted above, Noble and associates opined that such increases in aortic carbohydrates and mucoproteins comprised the earliest indications of aortic degeneration.

Schwartz and Gilmore (1958) have also recently related the serum mucoprotein and hexosamine levels in man to the existence of atherosclerosis in living subjects, and they noted elevations of these plasma fractions in ageing individuals. These workers attributed the increase in circulating SMP and hexosamine to depolymerisation of aortic connective tissue ground substance—a change which they considered to play an important role in the pathogenesis of human atherosclerosis.

Certain stimuli are known to produce elevations in SMP in experimental animals and in man. In particular, Engel (1952) showed in rats, that injections of parathormone were rapidly followed by a rise in SMP ; this he attributed to depolymerisation of the ground substance of the bones. Boomfield and Hayes (1952), in addition, showed similar mobilisation of ground substance from cutaneous connective tissue inducible by parathormone. Kushner *et al.* (1956) demonstrated that in man, too, parathyroid extracts were among the most potent substances tested by them in elevating serum mucoproteins, although they found that fever and intensive ACTH therapy could also exert similar effects.

As shown above, in our califcerol treated rats SMP values rose only several days after the first dose and only after the observed elevation of the serum calcium levels. These two findings may have been closely related, as Engel claimed for his parathormone-treated rats. On the other hand, histological studies revealed in our rats a widespread appearance of PAS-positive material in arterial walls simultaneously with calcification. The latter may have been due, in part at least,

to the increased avidity for calcium of these newly appearing carbohydrates in the calcifying tissues. For Reaven (1953) and Sobel (1955) have averred that metastatic calcification was facilitated by alterations and especially by any increase or depolymerisation of tissue mucoproteins. This may well be the case for our rats especially since metastatic calcium deposits in aortae, coronary and other arteries, gastric muscle and mucosa, kidneys etc. were consistently associated with localised increases in the PAS-positive material located in precisely the same sites as calcium deposition (compare Figs. 15–18; also Gillman *et al.*, 1957).

Which of the above two changes came first, however, we could not ascertain with confidence. However, we do believe that alterations in the connective tissue ground substances, in all the above sites, probably played some role in the localised binding of calcium in injured areas. However, tracheal, bronchial and other cartilages, which are always laden with mucopolysaccharides even in normal rats, fail to calcify early. Also impressive was the rapid disappearance of calcium from the heart muscle, coronary arteries, gastric mucosa, pulmonary alveolar wall, renal tubules and their basement membranes—sites which are usually calcified at one phase of the initial responses to calciferol but in which PAS-positive material persists after calcification has resolved. While minerals undoubtedly accumulated in polysaccharide-laden sites in all the above tissues, the same minerals equally could, and usually did, disappear later, even though the muco- and other polysaccharides were still present in increased amount in the injured tissues.

It therefore seems that the presence of MPS is not alone effective in promoting calcification of tissues, although these mucopolysaccharides may, under certain circumstances, perhaps facilitate the binding, not only of calcium, but also of iron and especially of fat (Gillman and Naidoo, 1957; Gillman, 1957; Gillman *et al.*, 1957; Gill[illegible] and Hathorn, 1958). Buck and his co-workers (1954; 1958) also consider th[illegible]eposition of lipids (in cholesterol-fed rabbits) to be facilitated by preceding mucopolysaccharide accumulations in affected tissues.

Moreover, it can be seen from Fig. 6 that the rise in aortic calcium was accompanied by a large increase in the phosphorus content. This is of interest in view of the recent finding by Selye (1958) that administration of mono-sodium phosphate sensitizes the rat aorta to the calcifying effect of dihydrotachysterol. Selye's results, together with our observations, indicate that phosphorus is apparently an essential requirement for aortic mineralisation under these conditions, *i.e.*, calciferol and dihydrotachysterol intoxication.

The maintained serum mucoprotein elevations in our rats may perhaps also reflect the persistence of some profound alteration in connective tissue metabolism induced even by a single short period of calciferol intoxication. Whether such persistent alterations in connective tissue metabolism were due to some direct initial action of the calciferol itself, or to indirect effects consequent on early renal injury, as suggested by Gillman and Gilbert (1956*b*), or to secondary involvement of various endocrine glands, still remains problematical.

Relatively well established by our studies, however, is that aortic, coronary and other lesions once initiated, even by a single insult, are progressive and culminate in medial sclerosis, cartilage and even bone formation in the aortic wall. The heavy accumulation of MPS in the injured media of the coronary, renal and other medium-sized arteries studied by us, seems to be closely bound up with the

persistence and progress of the initial lesions. It is our impression, based on both microscopic and chemical data, that a new set of connective tissue regulatory mechanisms are initiated after the single period of calciferol intoxication. It is to this new set of regulations that both the progressive connective tissue MPS increases and the elevated SMP levels are perhaps due. The basic nature of the physiological and tissue enzymic changes still require study. However, it seems that the presence and even the degree of activity of reparative and degeneratory processes in connective tissue, particularly in arteries, may perhaps be predicted from the level of circulating SMP. For with the supervention of vascular collagenisation, later in our experiments, the SMP levels dropped (compare Figs. 4, 7 and 8).

The study of this aspect of the problem should indeed be rewarding. Our own analyses of the SMP in pregnancy and the puerperium in African women (Gillman and Pillay, 1959) and in the baboon (Gillman, Pillay and Naidoo, 1959) add weight to the possibility that active resolution or involutionary changes, at least in the connective tissues of the female genital tract, are closely associated with elevations in SMP levels (Gillman and Pillay, 1959). Moreover, Schwartz and Gilmore (1958) stress the ease with which SMP levels can be raised when depolymerisation of connective tissue ground substance is induced even by a single hyaluronidase injection.

We anticipate that increases in connective tissue MPS content will yet be shown to be closely associated with profound interference with the regeneratory processes in connective tissue, such as we have shown to be the case in lathyrus intoxicated rapidly growing weanling rats (Gillman and Hathorn, 1958 ; 1959).

Finally, we should like to stress, as we have before, (Gillman *et al.*, 1957) that, in agreement with Gillman and Gilbert (1956) there are obviously local peculiarities in the metabolic activity of the connective tissues in different sections of the arterial tree which make vessels vary in their susceptibility to the injurious actions of large doses of calciferol. Only on such a basis can we explain the obvious wave of calcification and decalcification (or persistent calcium deposits) detected in the heart, coronaries, aorta, etc. encountered at different times after administering calciferol.

The presently recorded chemically determined sequence of events indicates that healing in blood vessels is comparable only in broad trends with that noted by others in healing cutaneous wounds (Dunphy *et al.*, 1956 ; Jackson, 1957). All are now agreed that, in cutaneous wounds, there is initially an increase in hexosamine and that only later is this followed by a progressive increase in the amount of collagen. Repair of aortic injuries seems to follow a similar but not identical course.

However, there are at least two important differences between aortic and cutaneous repairs. Thus, in cutaneous wounds, hexosamine increase is notable within 2–4 days of injury, a decrease following in a further 2–3 days ; increases in chemically determined collagen have been recorded from the 4th day onwards. However, the hexosamine content of the aorta rises a little only 10 days after the medial injuries have been inflicted, remains elevated thereafter, but shows an even further increase at 160 days after the injury, possibly in association with both cartilage and additional collagen accumulation in injured areas. Collagen increase is always far less in the aorta than in skin wounds and becomes impressive very much later (Fig. 30), rather than in 2–3 days as is the case for the skin. The very

much slower rate of fibrosis as well as delayed hexosamine and cartilage accumulation in traumatised aortic tissue has been fully documented histologically in several previous studies (Gillman *et al.*, 1957 ; Gillman and Hathorn, 1957 ; 1959 ; Gillman, 1959*a*).

We have stressed some of the possible implications of these observations elsewhere, for we regard them as basically important for understanding the pathogenesis of arterial diseases (Gillman, 1957 ; 1958 ; 1959*a* ; Gillman *et al.*, 1957 ; Gillman and Hathorn, 1957 ; 1958 ; 1959). Simply stated, it seems justifiable to suggest that intramural scar formation, subsequent endarterial fibrin deposits and mural thrombosis, with ensuing vascular occlusion, may, at least in some instances be only the very late end results of even single incidents of vascular trauma, inflicted very much earlier in the life of the individual. Our earlier experimental data (Gillman *et al.*, 1957 ; Gillman, 1957, 1958 ; Gillman and Naidoo, 1957) indicating that healing acute vascular injuries may facilitate lipoidal and other degeneratory changes in blood vessels, have also been fully substantiated recently (Constantinides *et al.*, 1958).

We have also adduced evidence to indicate that two separate types of arterial sclerosis probably occur in man. The first type may originate in the manner just outlined above, namely, the sclerosis ensuing even many years after the infliction of acute injuries to arteries. As suggested elsewhere such " injuries " may be initiated by physical or metabolic " traumata " and, especially during that period of active aortic remodelling which is probably essential for the growth of the vascular system accompanying a rapid total body growth characteristic of adolescence (Gillman and Hathorn, 1959 ; Gillman, 1959*a* ; 1959*b* ; Gillman and Hathorn, 1958).

This type of vascular scarring may perhaps be compared with Harris' lines in bones, the latter being attributed to disturbances of osseous growth and remodelling caused by intermittent illnesses in young actively growing individuals. If rapidly growing bone may be damaged (" scarred ") by intermittent illnesses, it does not seem unreasonable to propose that rapidly growing vessels may likewise be susceptible, even though such injuries to arteries are detected only much later on in life when they become manifest in many ways. For the effects of repair of such vascular injuries, if they are (as we suspect) inflicted during adolescence, would not be detectable until they had progressed sufficiently to cause functional circulatory impairment.

The recent epidemiological studies of Holman *et al.*, (1958) would seem to substantiate the above orientation originally proposed by us more than a year ago (Gillman, *et al.*, 1957 ; Gillman and Hathorn, 1957 ; Gillman, 1957 ; 1958). For, Holman *et al.*, (1958) confirmed, on a grand scale, the basic observations of Moon (1957) that the earliest stages of arterial degeneratory change are frequently present in the aortae of pre-pubertal and adolescent individuals. However, Holman and his associates stated that fibrotic plaques were not notable in the aortae of either Europeans or Negroes until 10 to 15 years after the first lesions were detectable, *i.e.* only in individuals aged 30–35 years.

These findings in man are in direct conformity with our previously recorded experimental facts and the hypothesis based thereon (Gillman, 1957 ; 1958 ; Gillman and Hathorn, 1957 ; 1958 ; 1959). This type of " post-traumatic " arterial fibrosis may indeed account for the frequency of fibrosing arterial degeneratory disease in relatively young adults. If rapidly progressive, and especially if

aggravated by dietary and metabolic factors—and particularly those determined by sex hormones (Gillman, 1958 ; Gillman and Naidoo, 1958 ; Gillman, Naidoo and Hathorn, 1958)—then such early lesions may perhaps explain the great frequency of advanced coronary and other vascular lesions in certain individuals during the fourth decade of life. The recent experimental findings of Constantinides and co-workers (1958) on the facilitation of lipoidosis and thrombosis by previous injury of vessels, also completely support our previously expressed views.

The second type of vascular sclerosis which we think occurs, is that which results from the gradual deterioration of the regenerative processes in vascular tissues into repair processes. For, as described elsewhere (Gillman, 1958 ; 1959*a*, *b*) sclerosis (scar formation) does not represent connective tissue regeneration, but rather that type of connective tissue neogenesis attendant on tissue destruction with the ensuing architectural distortion inevitably associated with scar formation (Gillman, 1959*b*). Such sclerosis may supervene when, with ageing or through disease, the normal mechanisms for regeneration of " wear and tear " changes in tissues fail. While this type of vascular sclerosis may perhaps be initiated at any time in life, we consider it to be probably most prominent in biologically aged or ageing systems (Gillman, 1959*a*, *b*). The vascular sclerosis found in those individuals who have lived to a ripe old age probably represents a combination of both of the above types of arterial repair (Gillman, 1958 ; 1959*b*).

SUMMARY

The object of this study was to determine the histological, histochemical and chemical changes induced in the rat aorta by toxic doses of calciferol, and to compare these with changes found histochemically in the heart muscle and coronary arteries. To this end the hexose, hexosamine, collagen, elastin, calcium and phosphorus contents of the aorta, and the serum calcium and mucoprotein levels were determined, in a total of 90 rats at intervals up to 160 days after a 5-day period of calciferol administration. There was an equal number of pair-fed control rats.

The mineralization (13–14 per cent Ca^{++}) induced in the aorta by calciferol persisted for a long period, and was not related to the serum calcium level, which dropped rapidly after calciferol administration was discontinued. This persistence of aortic mineralization contrasted markedly with the disappearance about 10 days after the completion of calciferol administration of histochemical evidence of mineralisation of the heart and coronary arteries.

Chemically, the laying down of calcium in the aorta was closely paralleled by a large increase in the phosphorus content.

There was a prolonged rise in the serum mucoprotein level which contrasted sharply with the transient rise in serum calcium.

Histochemically, there was a general increase in periodic-acid Schiff positive material in calcified tissues. Chemically there was a rise in total aortic hexose and hexosamine to approximately double the control levels ; these increases, however, were not quantitatively related to the rise in calcium content of the aorta.

The total aortic collagen appeared to increase towards the end of the experimental period, coinciding with the histological appearance of aortic and coronary

sclerosis. This supports the concept that healing in vascular wounds pursues a much slower course than cutaneous wound healing.

The implications of these findings for the understanding of the pathogenesis of atherosclerosis and vascular regeneration, degeneration and repair are discussed.

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CARDIAC LESIONS PRODUCED IN THE RABBIT BY INTRAMYOCARDIAL INJECTION OF VARIOUS MICRO-ORGANISMS*

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SEVERAL studies were undertaken in order to reproduce in laboratory animals cardiac lesions which would resemble those appearing in human beings suffering from the sequelae of streptococcal infections (Schultz, 1936 ; Smith, Morgan and Mudd, 1940 ; Gross, Cooper and Philips, 1941 ; Robinson, 1947 ; Schultz, 1947 ; Murphy and Swift, 1949 ; Murphy, 1949 ; Clawson, 1950 ; Robinson, 1951 ; Murphy, 1952 ; Glaser, Thomas, Morse and Darnell, 1956). These studies describe the histopathological changes obtained in laboratory animals following inoculation by various routes of haemolytic streptococci, of their products and of a number of other micro-organisms.

As to the mechanism involved in the production of cardiac lesions, two main theories have been proposed : (*a*) allergic phenomena, or production of auto-antibodies to the heart muscle (Rich and Gregory, 1944 ; Rich, 1946 ; Cavelty, 1947*a* and 1947*b*), (*b*) direct action of haemolytic streptococci and their toxins on the heart muscle (McLeod, 1953 ; Kellner and Robertson, 1954*a* and 1954*b*). Although both theories have been supported by some experimental evidence, the exact mechanism has not yet been clarified.

This study describes the histo-pathological changes obtained in rabbits as a result of single intramyocardial injections of haemolytic streptococci and their cell-free extract, as well as of other bacterial species, both related and not related to haemolytic streptococci. The possible mechanism involved, and especially the role of trauma to the myocardium, in inducing cardiac lesions, is described.

MATERIALS AND METHODS

Micro-organisms : The following bacterial species were employed : (*a*) *Streptococcus pyogenes*, group A, strain S84, type 3 (obtained from the State Serum Institute, Copenhagen). (*b*) A nonhaemolytic variant of strain S84 accidentally obtained by repeated passages of strain S84 on a synthetic medium (Ginsburg and Grossowicz, 1957). (*c*) *Streptococcus faecalis*, group D, isolated from human faeces. (*d*) *Lactobacillus casei* 7469. (*e*) *Bacterium coli*, strain B.

Micro-organisms (*c*), (*d*) and (*e*) were obtained from stock cultures of the Department of Bacteriology at the Hebrew University-Hadassah Medical School to whom we are indebted. Micro-organisms (*a*)–(*d*) were cultivated in brain-heart infusion broth (Difco), while (*e*) was cultivated in an ordinary bacteriological broth. Usually a 16-hr. culture grown at 37° was employed. The bacterial growth was washed 3 times with 20 ml. of 0·066 M phosphate saline buffer (pH 7·4). The washed bacteria were then resuspended in buffer and diluted

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to contain approximately 5×10^8 cells/ml. In some experiments streptococci were heated at 100° for 30 min., or irradiated fro 15 min. with ultraviolet light using a Mineralight model V 41 ultraviolet lamp, from a distance of 20 cm. No viable micro-organisms were left as determined by plating method.

Preparation of cell-free extract : Washed streptococci were subjected to sonic disintegration using a Raytheon 9 Kc sonic oscillator for 30 min. The cell debris and the remaining intact cells were removed by high-speed centrifugation and the supernatant fluid was filtered through a fine sintered glass filter.

Animals : Sixty-two male and female rabbits, weighing 2–3 kg., raised at the animal house of the Hebrew University, were used. They were kept on a vegetable diet and water, both given *ad libitum*.

Material injected : Amounts of 0·2 ml. of the above mentioned micro-organisms, or of cell-free extract obtained from strain S84, were injected into the myocardium of the unanaesthesized animals.

Histological preparations : The animals were killed at 7 and 14 days after the intramyocardial injection. Heart slices were fixed in Zenker's acetic acid solution. Paraffin sections were stained with haematoxylin-eosin, Van Gieson, azan stains, and Gram stain.

RESULTS

Group I (*Control animals*).—This group comprised 8 animals. The hearts of 4 rabbits were traumatized twice with a sterile needle (22 gauge), but no material was injected. In the other rabbits, sterile phosphate saline buffer was injected into the myocardium. The histological examination of heart sections in some cases revealed a proliferation of collagen fibres in the myocardium ; in others no pathological changes were seen.

Group II.—This group consisted of 27 animals. In 13 rabbits living streptococci (S84), in 5 rabbits heat-killed streptococci, and in 5 animals ultraviolet irradiated streptococci were injected into the myocardium. The remaining 4 animals received intramyocardial injection of cell-free extract obtained from strain S84.

The histological examination revealed pronounced endomyocardial changes in all the animals injected with any type of material. The intensity of the histological picture however varied (*a*) in different animals, (*b*) according to the number of days which elapsed from the injection to the time of sacrifice. The endocardium of animals killed 7 days following injection showed a marked fibroblastic and histiocytic proliferation which in some areas showed a palisade arrangement (Fig. 1 and 2). Mingled with these cells were some leucocytes. The endothelial lining of the endocardium was usually preserved and in some areas proliferation of these cells was marked (Fig. 3). The myocardial changes consisted of muscle degeneration and necrosis with a proliferative, granulomatous reaction. The latter consisted of foci of mononuclear cells, histiocytes, fibroblasts and giant cells with a basophilic or mildly eosinophilic cytoplasm. In the multi-nuclear giant cells, the nuclei were irregularly scattered in the cytoplasm and had a prominent, large nucleolus. The cytoplasm of some giant cells contained eosinophilic material. In some areas the granulomata surrounded empty spaces or some eosinophilic finely granulated fragments, which resembled sarcoplasmatic remnants (Fig. 4). The granulomata were often surrounded by lymphoid cells, and in some areas by a proliferation of collagen fibres. Similar histopathological changes were obtained by intramyocardial injection of a cell-free extract obtained from strain S84 (Fig. 5). In other areas no relationship between the location of the granuloma and the degenerated muscle fibres was found. No micro-

organisms were detected in the histological section or by culture. In animals killed 14 days following injection the histological picture was essentially similar to that described above, but the granulomatous lesions were more pronounced. In animals killed as early as 3 days following injection muscle necrosis and mononuclear cell infiltration could already be seen. It is of interest that only few granulocytes were present at that time.

Group III.—This group consisted of 9 animals : 3 animals received an intramyocardial injection of a mutant of strain S84. Enterococci, lactobacilli and *Bact. coli* respectively were injected into the myocardium of each 2 of the remaining 6 rabbits.

The histological changes in this group closely resembled those obtained in Group II. The most striking similarity was observed following the injection of *Bact. coli* and *L. casei* (Fig. 6) and a mutant of strain S84. In the case of the enterococci the lesions were of a milder degree. The lesions were abacterial.

Group IV.—This group consisted of 18 rabbits. Ten animals received a single intravenous injection of 1 ml. of living strain S84, but no previous trauma to the heart was performed. In another 8 animals the myocardium was traumatized twice with a sterile needle so as not to penetrate into the cavity of the heart, and 5 min. later 1 ml. of living strain S84 was injected intravenously into 6 of them, while the remaining 2 animals received 1 ml. of heat killed streptococci.

The histological examination of the heart of the animals receiving only an intravenous injection of living strain S84 showed no pathological changes. However, in 4 out of 6 animals the heart of which had been damaged by a sterile needle prior to the intravenous injection of living streptococci and in 2 animals receiving heat killed streptococci following needle trauma, well developed granulomatous lesions accompanied by muscle degeneration and necrosis were observed (Figs. 7 and 8). These alterations closely resembled those obtained in groups II and III. Of the remaining 2 animals, one exhibited interstitial myocarditis while the other revealed no pathological changes.

DISCUSSION

The results obtained in this study show that pronounced endomyocardial changes were found in rabbits at the site of a single intramyocardial injection of living or killed haemolytic streptococci, of their cell-free extract, and following injections of other micro-organisms. It is noteworthy that the histological changes obtained in the various groups examined were much alike. That the endomyocardial lesions produced by haemolytic streptococci could not be differentiated from those obtained by other micro-organisms indicates that the alterations are not specific for haemolytic streptococci. Myocardial changes similar to those described by us have been reported by Gross, Cooper and Philips (1941) following intracardiac injection of α-haemolytic streptococci. These authors claimed that the histological changes were identical irrespective of whether single or repeated injections were given. Glaser, Thomas, Morse and Darnell (1956) reported the appearance of myocardial granulomata 48–72 hr. following a single intrapharyngeal injection of haemolytic streptococci. These observations make it unlikely that sensitization or allergy play a significant role in their production. Since well developed granulomata have been found in our study 7 days after one single injection, the role of sensitization in the production of these lesions is

further questioned. As the injections of the various organisms caused trauma to cardiac muscles, the question arose as to whether or not trauma to the heart conditions the production of granulomata by the micro-organisms. This assumption was based on the fact that neither intravenous injection of streptococci nor the intramyocardial injection of buffered saline nor the puncture of the heart muscle itself by a sterile needle produced significant histological changes. However, fully developed muscle alterations and granulomatous reaction were observed in the myocardium following intravenous injection of living or dead streptococci when the rabbits' hearts were previously traumatized by a sterile needle.

These findings suggest that trauma to the heart is of importance in the production of myocardial changes, necrotic and granulomatous in nature, provided that streptococci are present in the blood. The possibility exists that products of various micro-organisms—toxins and enzymes—may cause " trauma " to the heart muscle, and thereby predisposing it to the effect of the micro-organisms. Kellner and Robertson (1954*a* and 1954*b*) showed that various proteolytic enzymes of streptococcal and other origin produced cardiac lesions in rabbits when injected intravenously. These lesions are similar to those obtained by other authors using whole streptococcal suspension (Murphy, 1949 ; Robinson, 1951 ; Glaser *et al.*, 1956). More recently it has been shown that various streptococcal haemolysins may injure and kill various mammalian cells *in vitro* and that these injured cells may be completely disintegrated when small amounts of various proteolytic enzymes are added to them (Ginsburg and Grossowicz, 1957).

It will be of interest to establish whether or not a combination of haemolysins and proteolytic enzymes may predispose the heart to develop such lesions.

SUMMARY

Histopathological changes obtained in the rabbit's heart by a single intramyocardial injection of haemolytic streptococci were studied.

EXPLANATION OF PLATES

The histological preparations were obtained from animals sacrificed 7 days following intramyocardial injection of the various micro-organisms.

FIG. 1.—Endomyocardial lesions following injection of haemolytic streptococci (strain S84). Note the fibroblastic and histiocytic cell proliferation in the endocardium mingled with round cell infiltration. A small granulomata with giant cells is seen adjacent to the endocardium. × 117. Haematoxylin and eosin.

FIG. 2.—Palisade arrangement of the proliferation in the endocardium following injection of haemolytic streptococci. × 406. Haematoxylin and eosin.

FIG. 3.—Proliferation of endothelial lining cells of the endocardium mingled with histocytes and round cells following injection of haemolytic streptococci. × 406. Haematoxylin and eosin.

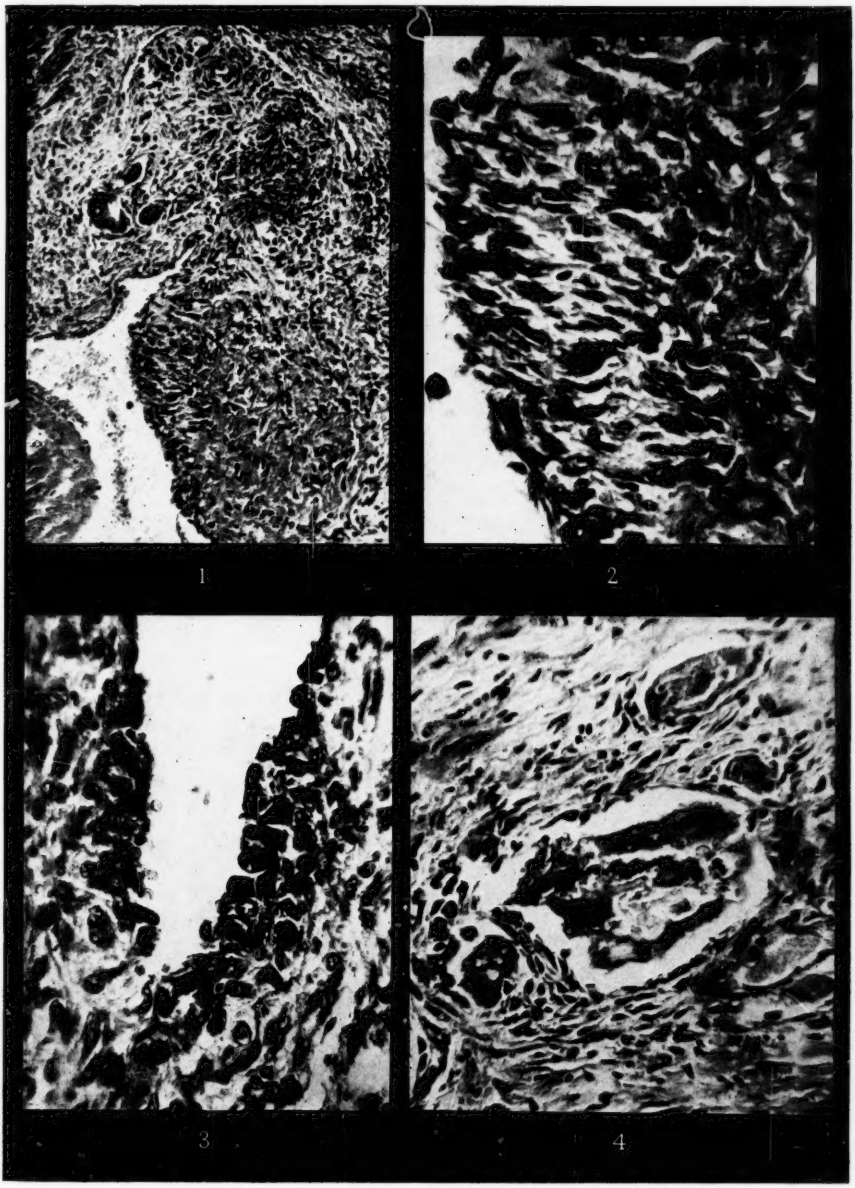
FIG. 4.—Granulomatous lesion in the myocardium surrounding necrotic muscle fibres following injection of haemolytic streptococci. × 406. Haematoxylin and eosin.

FIG. 5.—Granulomatous lesion in the myocardium surrounding necrotic muscle fibres following injection of a cell-free extract of haemolytic streptococci. × 114. Haematoxylin and eosin.

FIG. 6.—Granulomatous lesion in the myocardium following injection of *Lactobacillus casei*. × 285. Haematoxylin and eosin.

FIG. 7.—Granulomatous lesion in the myocardium obtained after cardiac damage with a sterile needle followed 5 min. later by an intravenous injection of haemolytic streptococci. × 107. Haematoxylin and eosin.

FIG. 8.—Same as in Fig. 7. Note the presence of mono- and multinuclear giant cells with large nucleoli. × 371. Haematoxylin and eosin.



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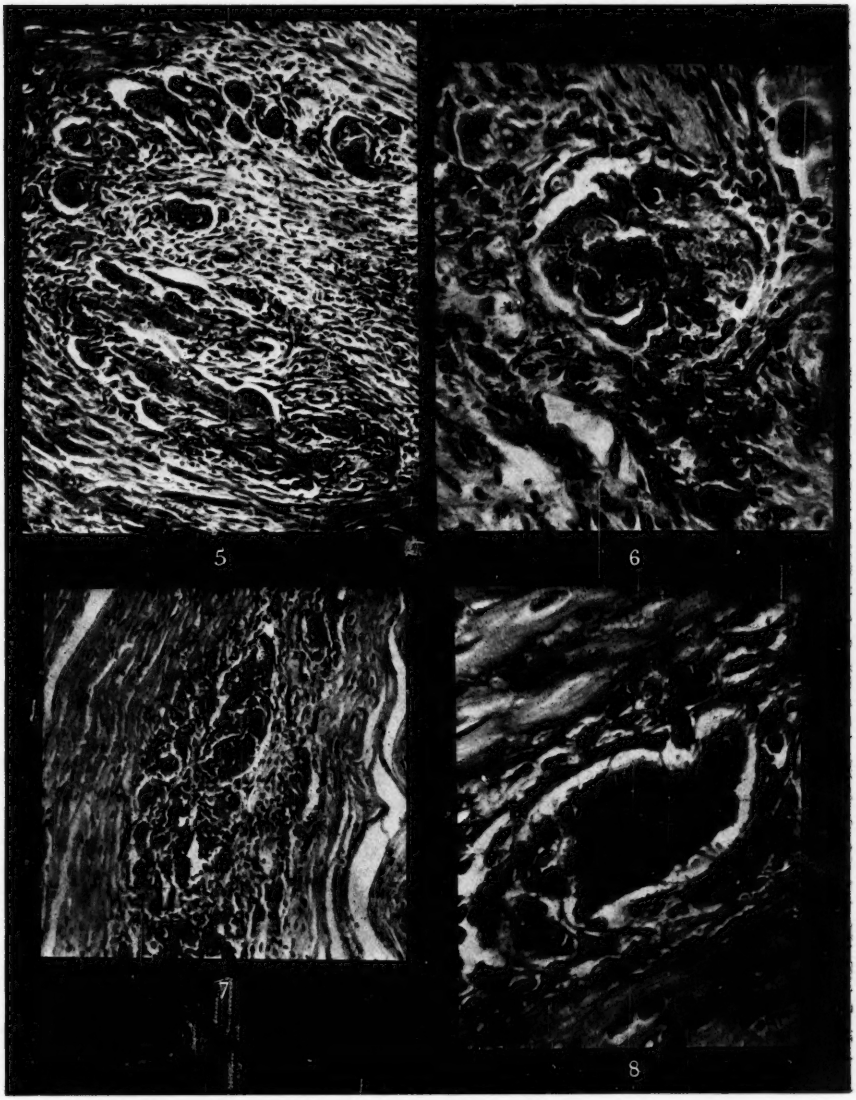

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Nonbacterial endomyocarditis accompanied by muscle necrosis and marked granulomatous reaction was observed.

Such lesions were not specific for haemolytic streptococci since similar lesions were obtained following intramyocardial injection of enterococci, lactobacilli, *Bact. coli* and a nonhaemolytic mutant of haemolytic streptococcus.

Neither trauma to the heart muscle alone, nor the intravenous injection of living or heat-killed haemolytic streptococci caused any pathological changes in the heart. On the other hand, well developed granulomatous lesions appeared in the heart when the intravenous injection of these organisms was preceded by puncturing the myocardium with a sterile needle.

The specificity of haemolytic streptococci and the role of trauma in the production of cardiac lesions is discussed.

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THE IMPORTANCE OF ANTIBODY IN THE PREVENTION OF EXPERIMENTAL CHOLERA IN RABBITS

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IT has yet to be satisfactorily demonstrated that immunization protects humans against cholera (Burrows, Mather, Elliot and Wagner, 1946). The presence of antibody (" Copro-antibody ") in the gut, of human volunteers has been demonstrated by Burrows and Havens (1948) and these workers have further shown that in specifically immune guinea pigs *Vibrio cholerae* is eliminated from the gut faster than in normal animals (Burrows, Elliot and Havens, 1947). However since the transient infection in guinea pigs in no way resembles human cholera in its pathology, it is difficult to draw any conclusions as to the role of copro-antibody in the human disease. Using the experimental techniques of De and Chatterje (1953) and Dutta and Habbu (1955), it has been possible to study in rabbits, an enteric infection produced by *V. cholerae* which in many ways resembles human cholera, and the effect of specific antibody on the course of this infection.

MATERIAL AND METHODS

Bacterial strains.—The strain of *V. cholerae* Ogawa 5596 was obtained from the National Collection of Type Cultures, Colindale.

Preparation of rabbit antiserum.—The antigen was prepared by the method of Gardner and Venkatraman (1935) and the scheme of immunization as recommended in Standard Methods (Wadsworth, 1927) adopted.

Haemagglutination studies.—In these experiments the methods of Davies, Crumpton, Macpherson and Hutchison (1958) and Crumpton, Davies and Hutchison (1958) were used.

Counts on specific bacteria in positive and negative loops.—Serial dilutions of the washouts were made in saline and plated out on MacConkeys agar. After overnight incubation a number of colonies were selected at random from each of the plates and tested against a 1/500 dilution of homologous antiserum (titre of 1/2000). By this means an estimate of the total number of viable organisms of the specific type could be obtained.

Macrophage techniques.—Experiments with macrophages were carried out according to the methods described by Whitby and Rowley (1959).

Operative procedure.—Ligated loops were prepared in rabbits weighing 1–1·5 kg. as given in a previous paper (Jenkin and Rowley, 1959). The operations on weanling rabbits followed the procedure of Dutta and Habbu (1955).

Tissue culture medium.—Medium No. 199 (Morgan, Morton and Parker, 1950) kindly supplied by the Biological Standards department of the M.R.C. was used. This medium contained no antibiotic and was adjusted to pH 7·2 by adding fresh 1 per cent sodium bicarbonate solution.

RESULTS

The effect of active immunization on the production of positive loops

A series of rabbits were immunized with a heat killed suspension of *Vibrio cholerae* Ogawa 5596. Five days after the last immunizing dose, ligated loops were prepared in the ileum of each rabbit and injected with 1 ml. of a growing broth culture of *V. cholerae* Ogawa 5596 containing 5×10^8 orgs./ml. A series of normal rabbits was similarly treated as controls. Twenty-four hours later the rabbits were killed and the loops examined. In the immunized rabbits, with one exception, the loops were all negative whilst in the control animals they were all positive, showing haemorrhagic necrosis of the intestinal epithelium and fluid distension (Table I). These results strongly suggest that antibody is involved in

TABLE I.—*The Effect of Active Immunization on the Production of Positive Ligated Loops of Rabbit Ileum by* V. cholerae *Ogawa* 5596

Rabbit	No. of positive loops	Haemagglutinating titre of serum
1	0/3	500
2	0/3	1,000
3	0/3	500
4	0/3	320
5	3/3	800
6	0/3	3,200
7	0/3	3,200
8	0/3	3,200
9	0/3	25,600
10	0/3	1,600

Six control rabbits gave 18/18 positive loops and the serum contained <10 haemagglutinating doses per ml.

the prevention of positive loops in the immunized animal. A count of the specific bacteria in some of the loops showed that even in the negative loops of the immunized animals, *V. cholerae* was still present, the total count varying from 5×10^7–5×10^8 in the negative loops compared with $2{\cdot}0 \times 10^{11}$ in the positive control loops. This confirms the previous findings (Jenkin and Rowley, 1959) that only in those loops where the bacteria multiply do the characteristic positive loops appear with haemorrhagic necrosis and fluid distension.

Passive transfer of immunity

A rabbit which had been actively immunized to produce a high titre Ogawa 5596 "O" antiserum was bled by cardiac puncture and the serum separated. Two normal rabbits were injected i.v. with 10 ml. of the immune serum. Three loops were prepared in each of these rabbits and injected with 1 ml. of a growing broth culture of *V. cholerae* Ogawa 5596 containing 10^8 bacterial/ml. Two other control rabbits were injected i.v. with 10 ml. of saline and treated in an identical fashion. Twenty-four hours later all the rabbits were killed and a small sample of blood taken from the heart to determine the circulating antibody titre. Examination of the ligated loops showed that only in the control rabbits injected intravenously with saline were positive results obtained (Table II).

TABLE II.—*The Effect of Passive Immunization on the Production of Positive Ligated Loops of Rabbit Ileum by* V. cholerae *Ogawa* 5596

Rabbit	No. of positive loops	Haemagglutinating titre of serum 24 hr. after injection of antiserum
1	0/3	3,200
2	0/3	3,200

Two control rabbits gave 6/6 positive loops. Serum contained <10 haemagglutinating doses per ml.

Specificity of the protection afforded by immunization

Since Jenkin (1959) had shown that the protein toxins from antigenically unrelated vibrios were immunologically related, and that these protein toxins were the protein components of their "O" somatic antigens it was of interest to see if active immunization of rabbits against a water vibrio would confer immunity to *V. cholerae*. It was found however, that even though a high titre antiserum was obtained against the water vibrio, these rabbits all gave positive ligated loops when tested with *V. cholerae* Ogawa 5596. These results suggest that specific antibody directed against the lipopolysaccharide component of the "O" somatic antigen is important in conferring protection.

The role of antibody in protection

There are 2 known important host defence mechanisms involving antibody. One is the highly complex humoral bactericidal mechanism involving the 4 components of complement, properdin, and magnesium ions (Wardlaw and Pillemer, 1956) in addition to antibody (Rowley and Wardlaw, 1958), and the other is a cellular mechanism. Recently it has been shown that macrophages take up and kill bacteria only if these have been pretreated with antibody or non-specific "opsonins" (Rowley and Whitby, 1959).

The role of complement + antibody

It was possible that under an inflammatory condition the components of the humoral bactericidal mechanism might leak into the lumen of the gut and together with antibody form a bacteriolytic system. This hypothesis was tested in the following way. Three ligated loops in each of 6 rabbits were injected with a growing culture of *V. cholerae* Ogawa 5596. Four hours later in 4 of the rabbits the loops were injected with 20 haemagglutinating doses of a specific "O" antiserum. In 2 of these rabbits, the ligated loops were crushed with forceps to allow bleeding into the lumen. Twenty-four hours later all the rabbits were killed and all loops were found to be positive. Since the ligated loops were positive even in those animals where bleeding into the lumen of the intestine had taken place (thus supplying all the components of the serum bacteriolytic system), it is likely that humoral components plus antibody play little or no part in the destruction of injected bacteria in the intestine of the immune animal. This was further substantiated by the finding that tissue extracts from the ileum were anti-complementary as was the fluid from a positive loop.

The role of macrophages and " opsonic " factors in the immune rabbit

Whitby and Rowley (1959) have shown that the amount of antibody required to sensitize bacteria so that they are rapidly ingested and destroyed by macrophages, is much less than that which may be measured by the most sensitive agglutinating and haemagglutinating techniques. Since the experimental techniques adopted by these workers allows one to detect very small amounts of antibody and opsonic factors, it was decided to use these sensitive methods to detect antibody in the gut lumen of immune rabbits.

Ligated loops were prepared in normal and in immune rabbits that had been immunized with a heated suspension of *V. cholerae* Ogawa 5596. All loops were injected with 1 ml. of a broth culture of Ogawa 5596 containing 5×10^8 bacteria/ml. Three hours later the rabbits were killed and the loops removed and washed in sterile saline. The ligatures were cut and the contents of the loop which were free from blood were washed out with 10 ml. of sterile broth.

The washouts were diluted 1/100 in sterile saline and 0·2 ml. of this diluent injected intraperitoneally into a series of mice. Mice were killed at intervals and the peritoneum washed out with 1·5 ml. of sterile heparinized 199 medium. If antibody is present in the lumen of the gut of the immune rabbits then the injected *V. cholerae* will have been sensitized and ingested and killed by the macrophages.

A count on the peritoneal washout (T) revealed the *in vivo* rate of removal of the injected bacteria. One ml. of the peritoneal washout was transferred to a Porter flask (Porter, Claude and Fullam, 1945). Counts on the Supernatant (S) after allowing 25 min. for the macrophages to settle and stick to the glass revealed the number of bacteria in the macrophages by difference (T–S). The percentage phagocytosis could then be calculated by 100(T–S)/T.

The results given in Table III indicate that the bacteria from the loops of intestine in the immune rabbits have been in contact with " opsonic " factors, probably antibody. The slower but definite removal of bacteria from the loops of normal rabbits by mouse macrophages is very interesting and the significance of this observation will be discussed later. As a control, the rate of removal of a

TABLE III.—*Removal by Macrophages in the Mouse Peritoneum of* V. cholerae *Ogawa* 5596 *Recovered from Ligated Loops of Normal and Immune Rabbits*

Time after injection (min.)	*V. cholerae* from ligated loops of normal rabbit			*V. cholerae* from ligated loops of immune rabbit		
	Total organisms in peritoneal washout (ml. (T))	Total organisms in supernatant from flasks (ml. (S))	Per cent phagocytosis	Total organisms in peritoneal washout (ml. (T))	Total organisms in supernatant from flasks (ml. (S))	Per cent phagocytosis
Start	75,000	14,900	80	4,500	300	93
10	52,500	15,000	69	300	300	—
15	41,500	18,500	53	30	<10	—
25	45,500	20,500	54	60	<10	—
30	41,000	5,300	87	30	10	—
40	36,000	12,000	66	0	<10	—
50	30,500	11,500	58	0	<10	—
60	21,000	7,800	63	30	<10	—
70	12,000	2,400	80	0	<10	—
80	6,300	2,850	55	0	<10	—

broth culture of *V. cholerae* was also studied. In this last experiment it was found that the number of organisms recovered from the peritoneum remained constant indicating that the bacteria were not removed by the mouse macrophages.

Protection afforded by antibody to experimental cholera in infant rabbits

It has been found possible to produce a cholera-like disease characterized by a profuse water diarrhoea in weanling rabbits (Dutta and Habbu, 1955). This seemed to offer an excellent experimental model for testing whether *V. cholerae* that had been pre-sensitized with antibody would set up an infection when introduced into the gut.

To a rapidly growing broth culture of *V. cholerae* Ogawa 5596, 10 haemagglutinating doses of antibody were added, and the culture kept at 37° for 60 min. Such a dilution of the antiserum was in itself non-bactericidal. Under ether anaesthesia 0·25 ml. of the pre-sensitized culture containing 10^8 bacteria/ml. was injected into the small intestine of weanling rabbits, 7 days old. As a control, some of the infant rabbits received 0·25 ml. of a culture of unsensitized *V. cholerae* containing the same number of bacteria/ml., whilst others were given 0·25 ml. of saline. The rabbits were selected at random from 4 litters of the same age. Observations extended over a week, weights were recorded and signs of diarrhoea noted. All of the infant rabbits injected with unsensitized *V. cholerae* were dead within 72 hr. of injection, whilst only one of the rabbits that had received sensitized bacteria died (Table IV).

Post mortem examination of the dead rabbits revealed the lower part of the ileum, caecum and upper portion of the large intestine distended with rice water fluid.

DISCUSSION

It is apparent from the results given in this paper that specific immunization of rabbits prevents the production of positive ligated loops when the immune animal is challenged with *V. cholerae*. Alternatively if *V. cholerae* is pre-sensitized with antibody before injection into the small intestine of weanling rabbits then these animals are also protected against the infection. Weanling rabbits injected with unsensitized *V. cholerae* die with signs of a cholera-like disease.

The experiments with mouse macrophages clearly show that " opsonins " probably antibodies, are present in the lumen of the gut. However, the amount of antibody must be small since in saline washouts of ligated loops from immune animals no specific antibody could be detected by haemagglutination techniques. Microscopic examination of the washouts from the ligated loops showed the presence of large numbers of mononuclear cells. It seems unlikely, since the contents of the loop are anti-complementary, that the components of the complement system play a major role in killing the injected bacteria. The important defence mechanism in the lumen of the gut of immune animals seems more likely to be a cellular one involving antibody and macrophages. The slower but significant take-up of *Vibrio cholerae* from the intestine of normal animals by mouse macrophages, as compared with the lack of removal of the same strain grown in broth, would suggest from the studies of Whitby and Rowley (1959) that non-specific " opsonins " are also present in the gut of normal rabbits. We would tentatively suggest that these non-specific factors might be important in protecting normal

TABLE IV.—*The Effect of Presensitizing* V. cholerae *Ogawa* 5596 *with Specific Antibody on the Production of a Cholera-like Infection in Weanling Rabbits*

Rabbit	Weight (g.) at start	Weight (g.) 72 hr. or at time of death*	Day of onset of diarrhoea	Result
Weanling rabbits injected with unsensitized *V. cholerae*				
1	81	85	2	D
2	68	67	1	D
3	132	114	2	D
4	76	70	1	D
5	77	76	1	D
6	126	110	1	D
7	124	119	1	D
8	103	92	1	D
Weanling rabbits injected with sensitized *V. cholerae*				
9	95	165	—	S
10	77	89	2	D
11	115	165	—	S
12	112	185	—	S
13	140	210	—	S
14	109	170	—	S
15	113	175	—	S
Weanling rabbits injected with 0·25 ml. saline only				
16	44	71	—	S
17	98	149	—	S
18	91	162	—	S
19	68	112	—	S
20	60	105	—	S

* = Where animals died, this occurred within 72 hr.
D = Death.
S = Survival.

individuals against an enteric infection. The differences in the levels of these factors might explain the differences in susceptibility between normal animals to enteric disease.

SUMMARY

Adult rabbits which have been specifically immunized against *V. cholerae* are resistant to an experimental cholera infection. Weanling rabbits, that have had *V. cholerae* pre-sensitized with antibody injected into the small intestine likewise show a high degree of resistance against the experimental enteric infection. Evidence is given which suggests that the important defence mechanism of the gut is one involving antibody and macrophages.

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A HUMAN SKIN CULTURE TECHNIQUE USED FOR CYTOLOGICAL EXAMINATIONS

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DETAILED analyses of the normal human chromosome complement has recently been made possible by the development of new techniques. Ford, Jacobs and Lajtha (1958) have studied the chromosomes of bone marrow cells incubated *in vitro* for a short period. Tjio and Puck (1958*a* and 1958*b*) and Chu and Giles (1959) have used long term monolayer cell cultures derived from other human tissues obtained by biopsy. All these workers have confirmed that there are 46 chromosomes (Fig. 4), and it can now safely be said that the normal human chromosome complement is well known. It is natural that the next step should be to examine the karyotypes of patients with congenital abnormalities of a kind which could be due to a gross chromosomal aberration rather than to a gene mutation. Jacobs and Strong (1959) and Ford, Polani, Briggs and Bishop (1959*a*) have reported cases of Klinefelter's Syndrome with 47 chromosomes and an XXY sex chromosome constitution. Patients showing another abnormality of sexual development, Turner's Syndrome (Ford, Jones, Polani, de Almeida and Briggs, 1959*b*), have been found to have 45 chromosomes and an XO constitution. Mongoloid idiots also have 47 chromosomes; in this case the extra chromosome is apparently a small acrocentric autosome (Lejeune, Gautier and Turpin, 1959*a* and 1959*b*; Jacobs, Baikie, Court Brown and Strong, 1959; Ford *et al.*, 1959*c*). In studying conditions like these, where surgical procedures are frequently not required, techniques such as that of Puck, Cieciura and Robinson (1958), which enable cultures to be established from small skin biopsies, are of particular value. The present paper describes a similar technique and preliminary results of its application to the study of the chromosomes of patients with congenital abnormalities of the type indicated above.

MATERIALS AND METHODS

Biopsy

The biopsies were carried out in Glasgow, Edinburgh, London and Harwell. So far 10 biopsy specimens have been received and successful cultures have been established from all of them. Nine were pieces of skin and the other was a piece of testis which was established in culture by Dr. P. M. Peacock of Glasgow and transferred to Harwell as a plasma clot culture. The skin samples were transferred to Harwell in small McCartney bottles containing either medium 199 or a human AB serum (70 per cent), glucose-saline (30 per cent) medium, and the primary cultures were set up there. The time lag from biopsy to culture varied from a few minutes to about 24 hr for some of the tissues from Glasgow. On one occasion a successful culture was established from tissue which had been kept for 72 hr

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(48 of these at 4°) after biopsy before primary culture was set up. The age of the donors varied from newborn to about 50 years. The amount of skin taken varied considerably but it was found possible to work with a piece of skin about 3 mm. by 4 mm. The methods of removing the skin also varied and I shall describe only that used at Harwell. After sterilizing a region of the ventral surface of the forearm, a small area was anaesthetized with "Parsetic" (Parke-Davis). A small fold of skin about 1 cm. from the site of injection was picked up with forceps and cut off with a scalpel. This was then placed in medium 199 at room temperature and transferred to the culture room.

Media

The following is a brief description of the various media currently in use during the culture procedures.

Plasma.—Fresh chicken plasma, without anticoagulants, is used in all cases.

Chick Embryo Extract (*CEE*) is prepared from 10 day old chick embryos in essentially the way described by Bryant, Earle and Peppers, (1953). Certain slight modifications have been made because of the smaller bulk handled. Hanks' saline (Hanks and Wallace, 1949) is used in place of Earle's saline. The resulting fluid is a sterile, cell-free ultrafiltrate and is stored at − 10° for up to 2 weeks.

Medium 199 (Morgan, Morton and Parker, 1950) is obtained from Glaxo Laboratories, Greenford, England, as a 10X stock solution and is diluted with glass distilled water immediately before use.

Human Serum.—Serum is drawn off from a bottle of clotted AB blood, spun for 30 min. at 2,500 r.p.m. and then filtered through a No. 03 porosity Selas filter candle, at a positive pressure of 10 lbs./sq. inch, to ensure that it is cell free. Serum is stored at − 10° for up to one month.

Trypsin-versene mixture is made up from 3 stock solutions. (*a*) Hanks' solution without the $CaCl_2$ and the $MgSO_4$. (*b*) Difco (Detroit, U.S.A.) Bacto Trypsin 1 : 250 made up as a 1 per cent solution in $Ca^{++}Mg^{++}$ free Hanks' solution the pH being adjusted to approximately 7·4 with N1 NaOH. (*c*) Diamino-ethane-tetracetic acid (disodium salt) from B.D.H., Poole, England, is made up as a 1 per cent solution in exactly the same way. Immediately before use these 3 solutions are combined to give a final concentration of 0·05 per cent of both trypsin and versene in $Ca^{++}Mg^{++}$ free Hanks' solution. The pH is then adjusted to approximately 7·4 with $NaHCO_3$ (0·35 g/l).

Growth Medium A consists of 20 per cent CEE, 20 per cent human serum and 60 per cent medium 199. This is used as the fluid phase of the primary cultures.

Growth Medium B consists of 10 per cent. CEE, 20 per cent. human serum and 70 per cent medium 199, and is used for all subcultures.

Culture

The tissue is cut into pieces about 1 mm square and embedded in a CEE : chicken plasma (50 : 50) clot, in a 5 cm Carrel flask. Normally 5 or 6 such pieces are placed in one flask.

Two ml of the growth Medium A is then added. The flask is flushed out with 5 per cent CO_2 in air, stoppered with a silicone rubber bung and placed in the incubator at 37°. Usually little or no growth occurs for about 3 days (longer in some cases). A few fibroblast-like cells are then seen to be emerging from the explant and thereafter this outgrowth expands fairly rapidly. Mitoses are frequently observed in this outgrowth. The fluid medium is changed routinely every 2 days but the plasma clot is not replenished unless the explant is in danger of coming loose from the glass. The cells virtually digest themselves out of the clot. When the outgrowth is judged to be adequate, usually after 10–14 days, the medium is removed and replaced with 2 ml. of the 0·05 per cent trypsin-versene mixture pre-warmed to 37°. After approximately 20 min. at this temperature the cells of the outgrowth have been released from the plasma clot. The explant is not noticeably affected by this procedure. The suspension of cells is centrifuged at 500 r.p.m. for 5 min. and the pellet of cells resuspended in growth medium. All the cells are transferred to a new Carrel flask. If there are many cells, an 8 cm. Carrel flask with 10 ml. of medium is used, and if there are few, a 5 cm. flask with 2 ml. of medium. The flasks are gassed, tightly stoppered and returned to the 37° incubator. The cells begin to settle down on the glass within 1 hr.

After a further 3–4 days a heavy growth of cells covers the bottom of the flask. The cells can be subcultured for cytological examination at this stage or, if they are in a small flask, the number of cells can be built up by further subculture before examination.

The routine subcultures are made as follows. The medium is replaced with 10 ml. of prewarmed trypsin-versene mixture and the culture incubated for 10 min. at 37°. The cells are, by then, in suspension and are spun down for 5 min at 500 r.p.m. They are resuspended in growth medium B and a 1/10 or 1/20 dilution is made into a new flask. (Note that a heavily grown culture contains approximately 10^5 cells/ml so that a newly inoculated culture has between 0·5 and $1{\cdot}0 \times 10^4$ cells/ml). The gas phase is adjusted and the culture reincubated. The medium is changed routinely 3 times per week and subcultures are made on the average once per week. These cultures may be kept going for at least several months at an undiminished rate of growth. There is some evidence of a slowing of the rate of proliferation after about 3 months in culture.

When subculturing for cytological processing the cells are put into suspension as before and a rather heavy incoculum (2×10^4 cells/ml) placed into 5 ml. of growth medium B in a 9 cm. petri dish that contains a number of cover glasses attached to the bottom by means of a tiny drop of clotted plasma. The cells are thoroughly mixed and the dish placed in a sealed vessel (an ordinary dessicator with built-in shelves is used, but any similar container would do), which is then flushed out with 5 per cent CO_2 in air and placed in a 37° incubator. The atmosphere in the vessel is kept moist by having an open dish of distilled water in it.

Cytological Procedures

It was found that a considerable wave of mitosis occurs about 24 hr after subculture. Colchicine is therefore added to the culture 22 hr after subculture in a final concentration of 0·0005 per cent. The colchicine is allowed to act for 6 hr, by which time a considerable number of mitoses have accumulated. The cells on the cover slips are then treated in roughly the way described by Rothfels and Siminovitch (1958). They are removed from the petri dishes and given 15 min. hypotonic treatment at 37° in 25 per cent strength Hanks' solution (prewarmed) in a Columbia staining dish. They are then removed and wafted face down over the open neck of a bottle of 2 per cent osmic acid for about 2 sec. only. The cover slip is then held face down in glacial acetic acid—absolute alcohol (1 : 3) fixative for 1 min and placed gently in the same fixative in a Columbia staining dish for 30 min. It has been found that this modification of the Rothfels and Siminovitch technique tends to keep the cell membranes unbroken and scattering of the chromosomes is therefore less likely to occur. Care must be taken, however, not to overdo the osmic acid vapour treatment or spreading of the chromosomes may be seriously impaired. The cells are then air dried and stained in lactic-acetic-orcein on a siliconed slide. (Natural orcein, 2 g. in 1400 ml. of a 50/50 mixture of 70 per cent lactic acid and 99 per cent acetic acid). When the stain has been sufficiently taken up (I find it convenient to leave them overnight) the cover slip is floated off in 45 per cent acetic acid, the cells dehydrated and mounted in euparal.

RESULTS

The results which have been obtained so far are shown in Table I. Fuller details of some of these cases have been, or will be published elsewhere. The 4 cases of Klinefelter's Syndrome all have cells with 47 chromosomes and a presumptive XXY constitution (Fig. 1). This confirms the recent reports by Jacobs and Strong (1958) and Ford *et al.* (1959*a* and 1959*c*). The first case is case 1 of Stewart (1959) who describes the clinical features of this patient. The one case of Turner's Syndrome (Fig. 3) was found to have 45 chromosomes and was interpreted to have an XO constitution in accordance with the observation of Ford *et al.* (1959*b*). The finding that the patient showing both Klinefelter's Syndrome and mongolism has 48 chromosomes (Fig. 2) confirms the report on a bone marrow sample from the same patient (Ford *et al.*, 1959*c*). The case of epiloia has a normal chromosome complement. Miss Jacobs has allowed me to

record that she has made a similar observation on the bone marrow of another patient also suffering from epiloia. Ford and Penrose (unpublished) have also confirmed this finding on 2 cases. The patient with acute blast cell leukaemia has also a normal chromosome complement. It is important to be sure of this point in view of the fact that her bone marrow was found to contain many cells with chromosome counts other than 46. This case is fully reported by Baikie, Court-Brown, Jacobs and Milne (1959). The cells of the anencephalic foetus were found to have 46 chromosomes which were entirely normal (Fig. 5).

THE TABLE.—*Summary of Cytological Examinations*

Case No.	Clinical condition	Chromosome counts						Phenotypic sex	Sex chromosomes
		<45	45	46	47	48	>48		
1	Klinefelter's syndrome	1	3	3	50	1	—	M.	XXY
2	,, ,,	—	—	—	33	—	—	M.	XXY
3	,, ,,	—	1	—	46	1	—	M.	XXY
4	,, ,,	—	—	—	40	1	1	M.	XXY
5	Mongol with Klinefelter's syndrome	—	—	1	2	41	1	M.	XXY
6	Turner's syndrome	—	46	—	1	—	—	F.	XO
7	Acute blast cell leukaemia	1	—	35	1	—	—	F.	XX
8	Epiloia	—	—	45	1	—	1	M.	XY
9	Anencephaly	6	7	58	6	—	—	F.	XX
10	Normal	—	—	39	1	1	—	M.	XY

DISCUSSION

Most of the cases noted here have been or will be reported individually in detail and for this reason I do not propose to discuss the clinical details of these cases or their genetical implications. The discussion will therefore be confined to the advantages and disadvantages of this technique and the interpretation of the results achieved by this method.

Several methods of establishing cultures of human cells have already been described but many of these are dependent on a supply of material obtained during surgical procedures. If it is desired to establish cultures, for any reason, from a large number of patients, it is essential to have a technique which uses tissues accessible without major surgery. The short-term bone marrow culture

EXPLANATION OF PLATE

FIG. 1.—Klinefelter's syndrome. Cell with 47 chromosomes from skin culture Orcein. × 750.

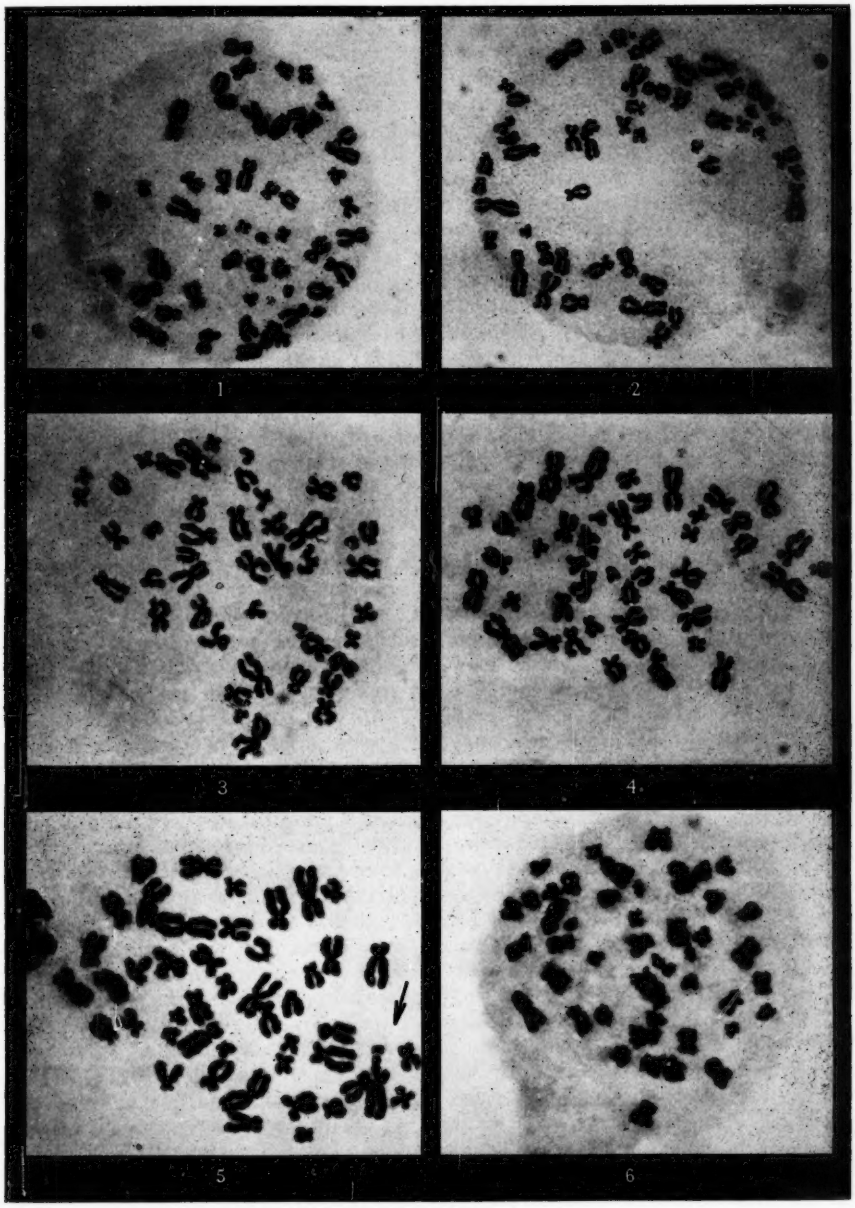
FIG. 2.—Mongol with Klinefelter's syndrome. Cell with 48 chromosomes from skin culture. Orcein. × 750.

FIG. 3.—Turner's syndrome. Cell with 45 chromosomes from skin culture. Orcein. × 1000.

FIG. 4.—Normal human female karyotype. Cell with 46 chromosomes from skin culture. Orcein. × 1000.

FIG. 5.—Chromatid break (arrowed) in a cell from an anencephalic female foetus. It is emphasised that the break has no particular significance for anencephaly. Orcein. × 1500.

FIG. 6.—Endoreduplication in a cell from a skin culture of a patient with Klinefelter's syndrome. Orcein. × 650.



Harnden.

method of Lajtha (1952) is useful for many purposes and has been used very successfully for chromosomal studies (Ford, Jacobs and Lajtha, 1958). Puck, Cieciura and Robinson (1958) report a very elegant technique for establishing long-term cell cultures from skin, using tryptic digestion as a means of getting the cells to disaggregate. The technique I have described was largely inspired by Puck's work but incorporates the basic idea of Harris (1955) who established long-term cultures of rat fibroblasts by growing the tissue for a short initial period in a plasma clot before tryptic digestion. The technique has been found to be very reliable. No piece of tissue received so far has failed to give rise to an actively growing culture. The fact that the tissue need not be dealt with for up to a few days after biopsy is of great value since tissues can therefore be sent long distances to the laboratory where culture is to take place. It is felt that the method should prove a useful tool especially at a time when the study of human cytogenetics is becoming rapidly more intensive.

The use of skin cultures for examining human chromosomes is not intended to replace bone marrow cultures for this purpose. It is, however, a useful alternative and, more important, the two techniques can be used together to give complementary data. This is of particular value in those cases where there is a possibility that the individuals are mosaics, *i.e.* made up of 2 or more clones of cells each with a different chromosome number. It is also important to know in cases such as the acute blast cell leukaemia described here, where the bone marrow contains cells which are grossly abnormal chromosomally, that the cells of the other tissues of the body contain a normal complement of chromosomes.

There are certain objections to the use of tissue culture for the examination of the chromosomes of any animal. The principal one is that the culture conditions may produce abnormalities which were not present in the original tissue (Levan and Biesele, 1958). A certain period must elapse from biopsy to the examination of the chromosomes. Both Tjio and Puck, and Chu and Giles give their minimum lag period as 1 week. Using the present method the average period elapsing before chromosome examination can be made is 2–3 weeks. The cultures when examined are almost entirely composed of euploid cells. A certain number of these are polyploid and some show endoreduplication (Fig. 6). Polyploidy and endoreduplication will not however obscure the original chromosome complement. A small number of cells with a chromosome count differing from the euploid number are found in most of the chromosome preparations (Table). Where the number is less than diploid, it is most likely that the cell membrane has broken and one or more chromosomes have been separated from the rest. One can most often see evidence of this rupture, particularly on examination under the phase contrast microscope, but the possibility that there are a number of truly hypodiploid cells cannot be eliminated. Although certain artefacts could suggest that an extra chromosome is present, some of the cells with more than the diploid number are probably truly hyperdiploid. Unless however several cells with the same modification are observed it is unlikely to be a significant deviation with respect to the nature of the original tissue. No such case has been noted but if it were one would still be uncertain whether this was an abnormality which had arisen in culture or whether it had been present in the original tissue.

The only other change to be noted is a small number of apparently broken chromosomes. Most of these "breaks" affect one chromatid only and may be

understained regions ("gaps") rather than true breaks. Dislocation of the fragment has however shown that one or two are true breaks (Fig. 5). There is a certain amount of evidence that the number of abnormalities increases as the culture ages. Certainly the number of polyploid cells increases in some cases quite markedly. The whole matter of chromosome abnormality in culture is at present under study. It should be noted however that Tjio and Puck have maintained cells in culture in the euploid condition for periods up to 10 months without any deviation at all from the normal.

Chromosomal studies on human abnormalities have so far produced a number of startling results in a very short period, namely the finding of individuals with one sex chromosome less than normal, with one extra sex chromosome and with one extra small autosome. It seems probable that other abnormalities will be discovered to be due to similar chromosomal aberrations. These need not be deviations in number but could be changes in the normal morphology of the chromosomes. Since the normal morphology is now well known an alteration in, for example, arm length or the position of the centromere in any particular chromosome might be detected by making suitable measurements. A preliminary examination of this kind on the case of epiloia failed to show any change.

SUMMARY

Long-term cell cultures have been established from small biopsies of human skin from several different individuals. From these, chromosome preparations have been made which enable detailed examination of the human karyotype. Several different human abnormalities have been studied. The finding of deviations in chromosome number in cases of Klinefelter's Syndrome, Turner's Syndrome and a patient showing both Klinefelter's Syndrome and mongolism confirm previous reports.

This work has been made possible by the cooperation of a large number of people. I should like to thank particularly my colleagues Dr. C. E. Ford and Dr. K. W. Jones, who have both encouraged me during the work and assisted me in the preparation of the manuscript, and Miss C. M. Scammell for her expert technical assistance. Material has been sent to me by Dr. P. M. F. Bishop, Dr. J. H. Briggs, Miss P. A. Jacobs, Dr. P. M. Peacock, Prof. L. S. Penrose and Dr. J. S. S. Stewart, with all of whom I have also had valuable discussions. The human AB serum has been generously supplied by the Oxford Centre of the National Blood Transfusion Service.

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V AND W ANTIGENS IN STRAINS OF *PASTEURELLA PSEUDOTUBERCULOSIS*

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STUDIES of the properties determining virulence in *Pasteurella pestis* showed that the potentiality for production of V and W antigens was, among others, essential for maximum virulence for mice and guinea-pigs. Thus all highly virulent strains were VW+ and all VW− strains were avirulent (Burrows and Bacon, 1956). Following the discovery of these antigens, a number of laboratory strains of *Pasteurella pseudotuberculosis* was subjected to conditions which, with *Past. pestis*, permitted V and W antigen production. No strain produced detectable amounts of them. It therefore seemed that V and W antigens were restricted to strains of *Past. pestis*, although the two species are well known to have many other antigens in common (Schutze, 1932; Crumpton and Davies, 1956; Thal, 1955, 1956; Ransome, 1956).

Isolates from guinea-pigs moribund during a natural epidemic of pseudotuberculosis differed from laboratory strains of the causative organism in producing readily detectable amounts of V and W antigens. One such isolate has been examined in some detail. Other strains of *Past. pseudotuberculosis*, recently isolated from naturally infected birds or mammals, have been investigated to determine the frequency with which VW+ strains occur in nature. Our results show that such strains are common and that the methods customarily used for culturing *Past. pseudotuberculosis* lead to selection against, and eventual elimination of, VW+ organisms to give VW− populations.

MATERIALS AND METHODS

Strains.—PB1, isolated in pure culture from a mesenteric lymph node of a guinea-pig during a natural epidemic of pseudotuberculosis, has received most study. Other strains, recently isolated from natural sources, were kindly sent to us. Details of the origin of all strains, together with some of their properties, are given in the Table.

Media.—Tryptic digest of meat broth (TMB) at pH 7 was used for liquid cultures. The addition of agar to 2 per cent final concentration in TMB gave the plating medium (TMA).

Cultures.—These were incubated at 28° or at 37° as specified later.

V and W antigens.—Conditions for V and W antigen production and detection were as previously described for *Past. pestis* (Burrows and Bacon, 1956). Briefly, organisms were incubated for 5 hr. in TMB at 37°, placed in reservoirs cut in agar plates and their soluble antigens allowed to diffuse against antisera prepared in rabbits using a VW+ strain of *Past. pestis* and absorbed with a VW − strain of this organism.

EXPERIMENTAL AND RESULTS

Strain PB1

This strain originated as follows. Enlarged mesenteric lymph nodes of guinea-pigs, during a natural outbreak of presumed pseudotuberculosis at Porton, were

sectioned and streaked on TMA. Independent isolates from 5 different animals yielded pure cultures of what appeared to be the same type of organism. One such culture was selected for further study and called PB1. It conformed with the published properties of *Past. pseudotuberculosis*. Unlike the laboratory strains of this organism examined previously, cultures of strain PB1 produced an antigen serologically indistinguishable from the V antigen produced by *Past. pestis*. The cultures however differed from those of the latter organism in producing only 1 of the 2 recognised components of W antigen. Using a system in which *Past. pestis* would be formulated as being V+, W1+, W2+, strain PB1 would be V+, W1+, W2—. For simplicity however strains will be called VW+ in this paper when they produce any of the V and W group of antigens.

When plated on TMA and incubated for 17 hr. at 37°, the original isolate of strain PB1 gave 2 colonial types ; a majority of smaller, perfectly circular, more opaque colonies and a minority of larger, slightly irregular, less opaque colonies. Cultures derived from single colonies of the former were all VW+, those derived from the latter were all VW—. Accordingly the 2 colony types were called PB1/+ and PB1/— respectively. On repeated subculture at 37°, PB1/+ always gave a proportion of /— derivatives, which were VW— ; in contrast PB/1— was never observed to give /+ colony types, or to be come VW+.

The distinction between /+ and /— colony types which was obvious when mixed platings on TMA were incubated at 37°, was not so apparent when incubation was at 28°, or lower. At the lower temperatures both types produced smooth, circular, equally opaque colonies. On 2 per cent peptone agar at 37° the colony types again were difficult to distinguish, both being smooth, circular and opaque ; the /+ types however showed somewhat more glistening surfaces than the /—.

To observe population changes involving the proportion of /+ to /— types during propagation *in vitro*, 2 cultures were initiated from one colony of PB1/+. The 2 were serially subcultured daily, on TMA slopes, one series being incubated at 37° and the other at 28° throughout. Daily, after transfer of inocula to the succeeding slope of each series, the remainder of the culture was tested for V and W antigen production and plated on TMA to determine the ratio of /+ to /— organisms. Cultures serially propagated at 37° became progressively enriched with /— type organisms so that after 9 serial transfers the original /+ type had become completely eliminated. Those propagated at 28° showed no evidence of selection against the /+ type over the period of the experiment. These results are illustrated in the Figure.

Cultures remained detectably VW+ until the proportion of /+ type organisms fell below 10 per cent of the whole.

Serial subculture at 37° of the series formerly being propagated at 28° showed similar selection against /+ organisms as had been observed with the 37° series above.

Other strains

The above observations suggested that our failure to detect V and W antigens in laboratory cultures of *Past. pseudotuberculosis* may have resulted from earlier elimination of /+ type organisms during culturing at 37°, the temperature at which this organism customarily is grown in the laboratory. Accordingly, we examined a number of strains, recently derived from natural sources, for their

ability to produce these antigens and for the presence of /+ type organisms, Many, and possibly all, the strains had been laboratory cultured to some extent. for identification, before being received and examined.

On receipt, the cultures were tested and scored as VW+ or VW− and plated on TMA. After incubation at 37° for 17 hr. the approximate percentage of /+ colony types was determined. Where cultures had been scored as VW− but were seen to give a small proportion of /+ type colonies on plating, cultures derived from these colonies were separately tested for V and W antigen production. If shown to be VW+, as was always the case, the original culture was classed as VW+. The results are shown in the Table.

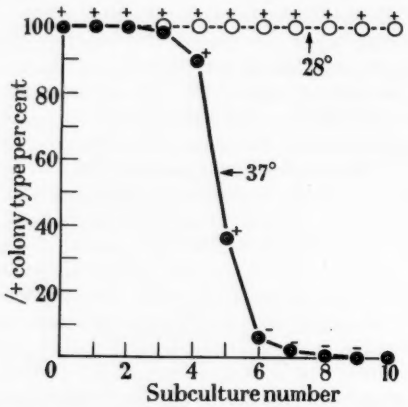

FIG.—Selection against /+ organisms during serial propagation at 37°, with resulting conversion of VW+ culture to VW−.

○----○ Series propagated at 28° on TMA slopes.
●——● Series propagated at 37° on TMA slopes.
+ = culture scored as VW+.
− = culture scored as VW−.

All strains were assigned to their serological Group on the basis of slide agglutination with Group-specific antisera ; strains were either of Group A or Group B, and no representatives of the relatively rare Groups C, D or E were received. (Groups A, B, C, D and E correspond respectively with Groups I, II, III, IV and V of Thal, 1954.) The /+ and /− components of strain PB1 both formed smooth suspensions in saline and were similarly agglutinable by Group A specific serum but not by others. Both therefore were smooth forms, in the serological sense (Davies, 1958), and did not respectively represent the smooth and rough forms of this organism. By the agar double diffusion procedure both could be seen to have Group A polysaccharide. In the many other strains examined, /+ and /− derivatives also had the same group-specificity as their parent culture. It will be seen from the Table that VW+ strains were common and could be either of Group A or B.

An incidental observation of interest, was that only strains of Group A were sensitive to the action of the bacteriocin-like material " pesticin ", produced

TABLE.—*Properties of Recently Isolated Strains of* Past. pseudotuberculosis

Strain	Received from	Isolated from	VW	Group	Per cent /+ colony type
PB1	—	Guinea-pig	+	A	60
218	S.V.A.	Hare	−	B	0
220	,,	,,	−	B	0
224	,,	,,	−	A	0
225	,,	,,	+	A	0·1
227	,,	,,	−	A	0
219	,,	Turkey	−	A	0
221	,,	,,	+	B	0·5
222	,,	,,	+	B	70
223	,,	,,	+	A	66
226	,,	,,	−	A	0
228	,,	Rabbit	−	A	0
679	V.I.C.	Pheasant	+	A	55
Z82	,,	Turkey	+	A	8
1342	,,	,,	−	A	0
1941	,,	,,	+	A	96
BB	,,	Bush-baby	+	A	100
M72	,,	Chinchilla	+	A	100
2096	,,	,,	+	B	50
21R	,,	Rabbit	−	A	0
K109	A.F.	Kale*	+	A	98
K111	,,	,,	+	A	40
K126	,,	,,	+	A	40
K129	,,	,,	+	A	10
K139	,,	,,	+	A	0·1
K160	,,	,,	+	A	90
K162	,,	,,	+	A	80
K163	,,	,,	+	A	70
K164	,,	,,	+	A	70
K165	,,	,,	+	A	60
K166	,,	,,	+	A	90
K108	,,	,,	+	B	10
K168	,,	,,	+	B	99
K106	,,	,,	−	A	0
S12	,,	Starling	+	A	95
295/A2	,,	Guinea-pig	+	A	99
E/A1	,,	,,	+	A	98
27/1128	U.T.	Man	−	A	0

S.V.A. = Statens Veterinärmedicinska Anstalt, Stockholm, Sweden.
V.I.C. = Veterinary Investigation Centres, Ministry of Agriculture, England.
A.F. = Allington Farm, Ministry of Supply, Wiltshire, England.
U.T. = Hygiene-Institut, Universität Tübingen.

* Pigeon faeces on kale in the field were cultured and injected into guinea-pigs and isolates from these animals sent to us.

by *Past. pestis* (Ben-Gurion and Hertman, 1958). Both VW+ and VW− derivatives of these strains showed sensitivity.

All VW+ strains of Group A were, like strain PB1, deficient in W2 antigen. The VW+ strains of Group B however were not ; such strains produced a complement of V and W antigens which could not be distinguished from that shown by VW+ strains of *Past. pestis*, *i.e.* they were V+, W1+, W2+. As observed with *Past. pestis* strains of *Past. pseudotuberculosis* either produced the full complement of V and W antigens characteristic of their Group or else produced none of them.

Single /+ type colonies of six different strains were serially subcultured at 37° to test whether, as had been seen with strain PB1, progressive enrichment of the cultures with /— types would occur. In all cases such enrichment was observed. From being initially 100 per cent /+ the strains K111, K108, K109, 295/A2 and K168 became 90 per cent /— type within 4, 5, 6, 9, 13 and 14 subcultures respectively. The variation in the number of subcultures producing population changes of this magnitude appeared mainly to result from variation in the time for the first appearance of /— types. Once such types arose they were rapidly selected.

DISCUSSION

It is difficult to decide to what extent our /+ and /— colony types conform with those described by others who have investigated variation in *Past. pseudotuberculosis*. Kakehi (1915) described colony variants arising in cultures which originated from a selected single colony representing the majority type of his initial culture. This colony, called type " A ", gave a proportion of type " B " colonies which in turn gave types " A ", " B " and a new type " C ". Type " C " gave rise to types " A " and " B ". Thus all types described by Kakehi appeared to be interconvertible, in contrast to our /+ and /— colony types. Two strains were studied by Zlatogoroff and Moghilewskaja (1928). After prolonged incubation in liquid media at 30–33° cultures were plated on peptone agar and yielded a variety of colony types, which varied in morphology according to the brand and quantity of peptone used. It is difficult to differentiate /+ and /— colony types on peptone agar incubated at 37°, and even more difficult on this medium incubated at lower temperatures. It is possible therefore that the " lisse S " colonies of these authors may be comparable with our types, but clearly the latter are not comparable with their " ridee R " colonies which form unstable suspensions in saline and granular growths in liquid media. Boquet (1937) did not specify the medium on which he observed " S " and " R " colony types but, since peptone medium was used for liquid cultures, we presume his solid medium to have been peptone based. His " S ", or " homogène ", type would resemble both /+ and /— types on this medium at 37°, but his " R ", or " agglutinable ", type would resemble neither.

Our limited survey of recently isolated strains of *Past. pseudotuberculosis* has shown that VW+ strains are of common occurrence in nature ; 27 of the 38 strains examined being VW+. Since selection against VW+ organisms occurs under conditions customarily used for the laboratory culture of this species, and since all strains had been subjected to such conditions before being received by us, it is probable that VW+ strains occur even more commonly. Most likely strains 225, 221 and 139 (Table) would have been scored as VW—, had they been further cultured under these conditions before being examined here. The selective pressure against VW+ organisms in *Past. pseudotuberculosis* on incubation at 37° resembles that demonstrated by Fukui, Ogg, Wessman and Surgalla (1957), for *Past. pestis*.

This work has produced yet another illustration that fundamental change from its state in nature may result during brief culture of a pathogen under laboratory conditions. In the closely related species *Past. pestis*, comparable change would result in conversion from high virulence to avirulence. It is already

apparent (unpublished observations) that VW— strains of *Past. pseudotuberculosis* have considerably reduced virulence for mice as compared with their VW+ counterparts. Propagation of this organism at temperatures not exceeding 28° would help, by avoiding gross population changes, to ensure that laboratory studies of experimental pseudotuberculosis have wider application to the naturally occurring disease. It would be of interest to know whether VW+ types are frequent among the strains of *Past. pseudotuberculosis* which are becoming increasingly recognised as associated with alimentary infections in man (Knapp, 1954; 1959). We have examined nine cultures of human origin, but not of recent isolation, and found all to be VW— ; freshly isolated cultures may well be otherwise.

Past. pseudotuberculosis and *Past. pestis* have many antigens in common and clearly have close taxonomic relationship. The similarities of the 2 species have led many to hypothesise that the former may, in some way, be concerned in the endemicity of plague. The sporadic derivation of *Past. pestis* from *Past. pseudotuberculosis*, an organism having wide geographical and host range, would nicely explain the otherwise puzzling alternation of plague epidemic and quiescence throughout history. Our finding that antigens essential for high virulence in the plague organism are possessed by many strains of *Past. pseudotuberculosis* in nature lends support to the hypothesis.

We are very indebted to Dr. E. Thal, Stockholm, Sweden ; Dr. W. Knapp, Tubingen, Germany ; Dr. A. W. Stableforth, Dr. E. T. Davies, Dr. H. E. Roberts and Dr. T. F. Varney of the Ministry of Agriculture and to Dr. J. S. Paterson and Mr. R. Cook of Allington Farm, Porton, for so kindly sending strains, and to Dr. D. A. L. Davies of M.R.E. for samples of specific polysaccharides. We would like to thank Dr. D. W. Henderson, F.R.S., for his interest and advice and Miss Barbara Alkins and Miss Dorothy Burrell for their excellent technical assistance.

SUMMARY

Many strains of *Past. pseudotuberculosis* recently isolated from natural infections show V and W antigens which characterise all fully virulent strains of *Past. pestis*. Of 38 such isolates, 27 were VW+. The majority were of serological Group A (22 VW+, 9 VW—), the remainder of Group B (5 VW+, 2 VW—). VW+ strains of Group A lacked one component of the *Past. pestis* VW antigen system ; those of Group B did not. Only Group A strains were sensitive to pesticin. VW+ strains usually were mixed populations of VW+ and VW— organisms, which gave distinguishable colonies on TMA at 37°. Repeated subculture at 37° resulted in selective elimination of VW+ organisms. Population changes were avoidable by maintaining and propagating cultures below 28°.

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THE INHIBITION BY DIOXAN OF ANTIGEN-ANTIBODY PRECIPITATION

II. Precipitin Reactions with Polysaccharide Antigens

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In a previous communication (Grant, 1959), it was shown that dioxan exerts an inhibitory effect on precipitin reactions between protein antigens and rabbit antibodies. This study has now been extended to include two reactions involving polysaccharide antigens. In view of the completely different chemical structures of polysaccharides and proteins, it was expected that the precipitin reactions of these materials would not be affected by dioxan in the same manner. This has been found to be so. Type III pneumococcal polysaccharide was chosen because the structure is known. It is composed of glucose and glucuronic acid molecules linked alternately through β-1-4 glycoside bonds and may be regarded as a linear polymer of cellobiuronic acid (Hotchkiss and Goebel, 1937). The structure of Type I pneumococcal polysaccharide is not completely known but it contains D-galacturonic acid and an acetylated amino sugar. Both of these polysaccharides are acidic. Since horse and rabbit antibodies behave somewhat differently, horse antiserum to Type I pneumococcus was included for the sake of completeness. The results presented here indicate that the mechanism of the precipitin reactions of these polysaccharides differs from that in which the antigen is a native protein.

MATERIALS AND METHODS

Methods.—These are as previously described (Grant, 1959).

Type I pneumococcal polysaccharide.—This was prepared by Mr. R. G. S. Johns by the method of Heidelberger, Kendall and Scherp (1936).

Type III pneumococcal polysaccharide.—This was obtained from Lederle Laboratories, lot 17.

Rabbit antiserum to Type III pneumococcus.—This was provided by Dr. R. G. White.

Horse antiserum to Type I pneumococcus.—This was prepared at the Lister Institute; it contained a little phenol as preservative.

RESULTS

The turbidity-time curves for both reactions (Fig. 1 and 5) show that the initial rise of turbidity was almost unaffected by dioxan. The second stage of the reaction, *i.e.* the formation of visible particles and precipitation was markedly inhibited, the tendency to precipitate diminishing as the dioxan concentration increased. Ethanol (20 per cent) somewhat increased the rate of development and the intensity of the turbidity and did not inhibit precipitation. In the case

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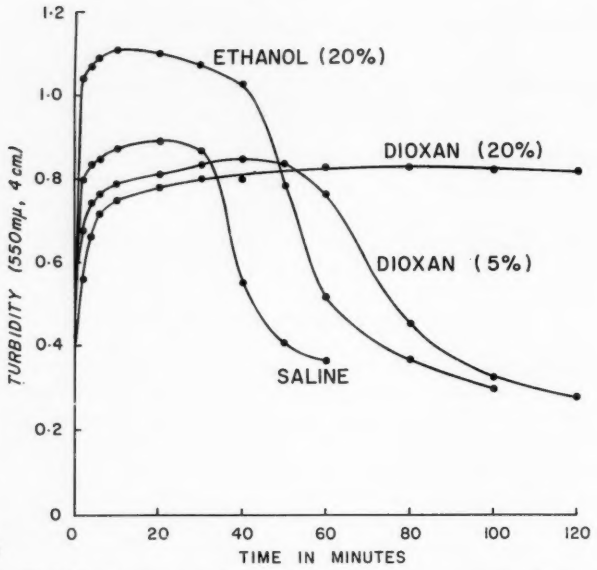

FIG. 1.—Turbidity-time curves for reaction of equivalent amounts of pneumococcal polysaccharide Type I and horse antiserum.

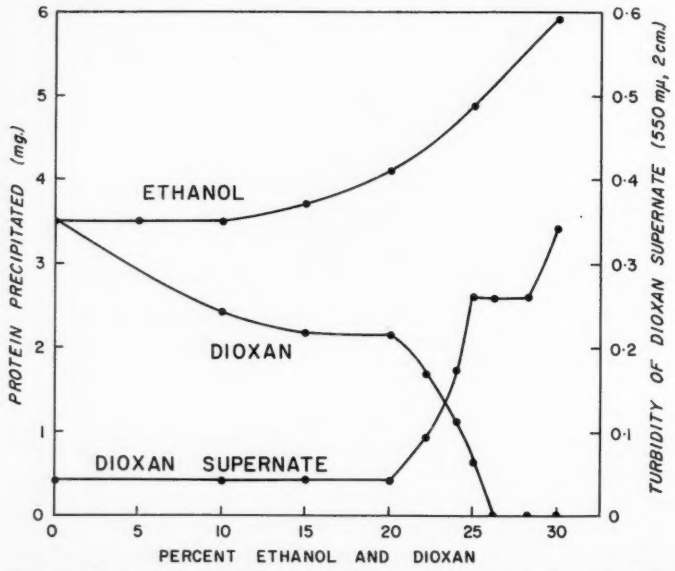

FIG. 2.—Comparison of effects of dioxan and ethanol on the amount of precipitate formed from pneumococcal polysaccharide Type I (0·25 equiv.) and horse antiserum (1 ml. in a final volume of 10 ml.).

of pneumococcal polysaccharide Type I and horse antiserum, dioxan concentrations of 26–30 per cent completely inhibited precipitation and stable turbid solutions were produced (Fig. 2). These turbid solutions showed little tendency to precipitate even on prolonged standing in the cold, and the turbidity values showed little change with time (Table). In contrast, ethanol in concentrations above 10 per cent caused an increase in the amount of precipitate, this being probably due to the formation of insoluble denatured protein. The degree of inhibition varied with the antigen-antibody ratio (Fig. 3), and was least in the

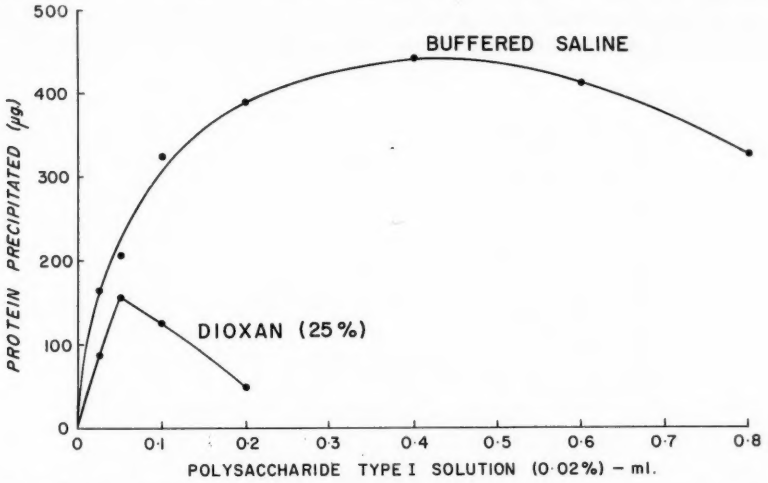

FIG. 3.—Variation of degree of inhibition with antigen-antibody ratio, pneumococcal polysaccharide Type I and horse antiserum.

zone of antibody excess. High concentrations of ethanol (20 per cent and above) caused delayed flocculation in both reactions, but standing in the cold (2°) rapidly induced precipitation, in contrast to similar solutions containing dioxan which were stable in the cold.

TABLE.—*Change of Turbidity with Time, Pneumococcal Polysaccharide Type I—Horse Antiserum—Dioxan, pH* 6·9

(Final dilution of antiserum, 1 : 10.)

Dioxan (per cent)	24 hr. at room temperature + 48 hr. at 0°	7 days at 0°	14 days at 0°	21 days at 0°	28 days at 0°
22	0·09	0·07	0·07	0·10	—
24	0·17	0·18	0·16	0·15	0·20
26	0·25	0·28	0·30	0·29	0·32
28	0·24	0·28	0·33	0·34	0·38
30	0·33	0·43	0·56	0·60	0·68

The amount of precipitate formed from pneumococcal polysaccharide Type I and horse antiserum is known to be reduced in the presence of high salt concentrations. Fig. 4 shows that in the presence of dioxan (10 per cent) the amount of precipitate formed was rather less than in a similar mixture containing no dioxan. The effect of a similar concentration of ethanol was also studied. With 0·15 M NaCl the addition of ethanol resulted in a slight increase in the amount of precipitate but as the salt concentration increased, the amount of precipitate fell, the effect being similar to that produced by dioxan.

When a solution of horse antiserum containing dioxan (25 per cent) was allowed to stand for 4 hr. at room temperature and then dialysed to remove the dioxan, the antiserum was found to flocculate more slowly than untreated serum.

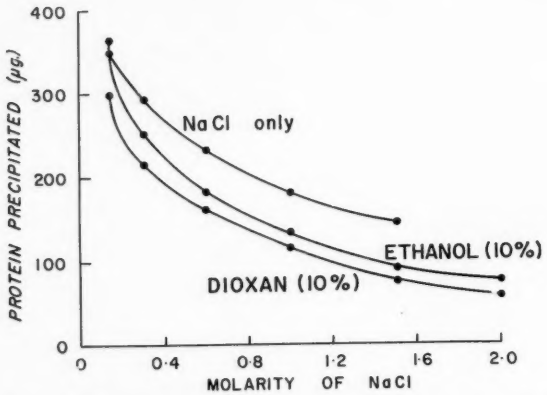

FIG. 4.—Comparison of effects of dioxan and ethanol on the dissociation of the precipitate from Type I pneumococcal polysaccharide and horse antiserum in the presence of high concentrations of salt.

Type I polysaccharide similarly treated showed no alteration in properties. A solution containing antigen, antiserum (final dilution 1 : 10) and dioxan (25 per cent) did not precipitate during 4 hr. at room temperature, but when the dioxan was subsequently removed by dialysis, a precipitate settled out leaving a cloudy supernate.

The final amount of precipitate from Type III polysaccharide and rabbit antiserum was reduced in the presence of dioxan (20 per cent) by only a small amount from 570–410 μg. protein. This system was the least sensitive to dioxan.

DISCUSSION

The results obtained from the reactions involving pneumococcal polysaccharide antigens were strikingly different from those found with the protein antigen systems. The turbidity-time curves for the two reactions (Fig. 1 and 5) indicate that both the rate of reaction as judged by the speed of development of turbidity, and the extent of reaction as indicated by the final turbidity value, were almost unaffected by dioxan. The flocculation times were greatly increased, and at certain concentrations of dioxan (25–28 per cent) precipitation was completely

inhibited, and stable turbid solutions resulted (Fig. 2). These turbid solutions showed no tendency to precipitate at 0° even after several weeks standing, the turbidity values showing little change with time (Table). It appears therefore that dioxan does not affect the initial phase of the reaction, the inhibitory effect being confined to the stage of flocculation and precipitation. The amount of precipitate was reduced over a wide range of antigen-antibody ratios, the degree of inhibition being least in the zone of antibody excess.

Ethanol showed no inhibitory effect on Type I polysaccharide-horse antiserum system (Fig. 1 and 2) ; instead, with concentrations above 10 per cent the amount of precipitate was increased. This indicates that inhibition by dioxan is not due to a lowering of dielectric constant. The results of the dialysis experiments

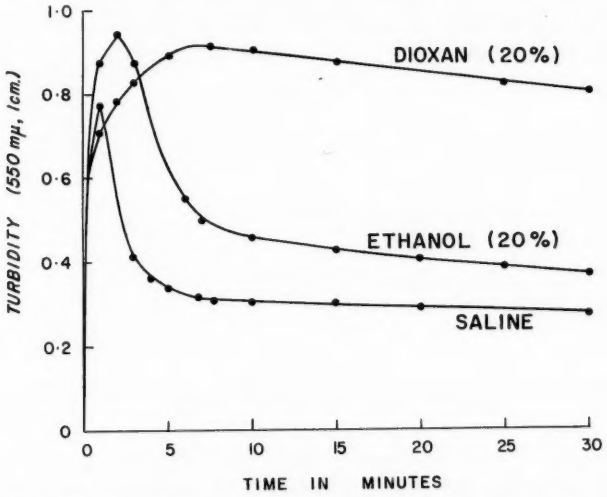

FIG. 5.—Turbidity-time curves for the reaction of equivalent amounts of pneumococcal polysaccharide Type III and rabbit antiserum.

showed that dioxan permanently decreased the precipitating power of the antiserum. The treated serum, however, was still capable of reacting with the polysaccharide since the mixture became turbid in a few minutes. Under the same conditions the polysaccharide remained unaltered.

There is a good deal of evidence that the reaction between an acidic polysaccharide and antibody is mainly ionic in nature. The precipitate is dissociated by high salt concentration (Heidelberger, Kendall and Teorell, 1936) while the precipitate from a native protein and antibody is not (Marrack and Höllering, 1938). Additional evidence is provided by the fact that antisera to pneumococcus Types III and VIII cross react with proteins coupled to glucuronic acid (Goebel, 1936), and antisera to Type II pneumococcus react with plant gums containing uronic acids (Heidelberger, Avery and Goebel, 1929). Also, Type III pneumococcus antisera precipitate with synthetic antigens consisting of proteins coupled with diazotised *p*-amino-phenyl carboxylic and sulphonic acids (Goebel and Hotchkiss, 1937). Esterification of the uronic acid group of Type I poly-

saccharide abolished the reaction with antiserum (Chow and Goebel, 1935). There is no evidence that similar ionizable groups form essential parts of the determinants of natural protein antigens. Marrack and Orlans (1954) found that acetylated bovine serum albumin could still react with antibody prepared against the unmodified albumin, and this has recently been confirmed by Houghton and Martin (1958). We should therefore expect that oppositely charged $—N^+H_3$ groups formed essential parts of the receptors of pneumococcal antibodies, but are not necessarily essential to the receptors of anti-protein antibodies. Evidence for this, based on the effects of formaldehyde, is not entirely satisfactory since formaldehyde appears to have other effects, *e.g.* aggregation, on protein molecules, in addition to combining with amino groups. Nevertheless, Eagle's (1938) results lend support to the foregoing arguments. He found that when horse antitoxin was treated with formaldehyde, flocculation was inhibited, but combination with antigen still occurred as shown by guinea-pig protection tests, even though sufficient formaldehyde was added to combine with all the free amino groups. In the case of horse antipneumococcus serum (to Types I and II), however, not only was precipitation and agglutination abolished, but the ability to combine with antigen was also destroyed, as shown by the failure of the treated serum to protect mice against pneumococci.

If the primary combination of pneumococcal polysaccharide and antibody depends on attractions between suitably situated $—CO_2^-$ and $—N^+H_3$ groups, then the lowering of dielectric constant due to dioxan will tend to increase these electrostatic forces. Also, charged groups such as $—CO_2^-$ and $—N^+H_3$ tend to form salt bridges rather than hydrogen bonds as is the case with hydroxyl groups. Hence it is possible that the anti-pneumococcal polysaccharide receptors, which appear to consist mainly of $—N^+H_3$ groups, are not blocked by dioxan as we assume is the case with anti-protein receptors containing hydrogen bonding groups such as the hydroxyl group of tyrosine. Dioxan would not therefore be expected to inhibit the primary combination of polysaccharide and antibody. Since the flocculation stage in the reactions of both Type I and III polysaccharides (Fig. 1 and 5) with the corresponding antisera was affected by dioxan, the mechanism appears to differ from that of the first stage. The fact that ethylene glycol and glycerol also delayed flocculation supports the idea that the second stage of the reaction involves hydrogen bonding (Grant, 1953).

Dioxan increases the degree of dissociation of the precipitate by high salt concentration (Fig. 4). We infer that inhibition by high salt concentration is due to competition by cations for negatively charged groups in the polysaccharide. This effect would appear to depend on the reduced dielectric constant of the solution due to the presence of dioxan since ethanol produced almost the same effect as dioxan under these conditions.

It is difficult to draw conclusions which have a direct bearing on the validity of the framework or other hypotheses of the mechanism of antigen-antibody reactions (Marrack, 1938 ; Boyd, 1947). Dioxan appears to differentiate between the first and second stages of reaction in the case of the pneumococcal polysaccharide antigen systems ; this is consistent with the concept that the first stage is mainly an electrovalent process. The dioxan-sensitive second stage may involve either specific or non-specific hydrogen bonding. With the protein antigen systems the first stage of reaction appeared to be inhibited, indicating that this phase involves hydrogen bonding ; this is not at variance with the

findings of other investigators. According to Goldberg's mathematical treatment of the framework hypothesis (1952), the size of the antigen-antibody aggregates diminishes in both the antigen excess and the antibody excess zones. Since dioxan inhibits most strongly when there is an excess of antigen, it would appear that dioxan acts mainly on the antibody.

SUMMARY

Dioxan has been shown to exert an inhibitory effect on two precipitin reactions in which pneumococcal polysaccharide antigens reacted with rabbit and horse antiserum.

The first stage of both reactions appeared to be relatively unaffected by dioxan, but the second stage of flocculation and precipitation was delayed or inhibited.

The results appear to support the concept that in this type of reaction the first stage of combination between antigen and antibody depends on attractions between oppositely charged groups.

The mechanism of inhibition of flocculation appears to depend on the hydrogen bonding properties of dioxan rather than on the lowering of dielectric constant of the solution.

In the presence of high concentrations of salt, the amount of precipitate formed (using Type I polysaccharide and horse antiserum) was less in the presence of dioxan than in a similar solution containing salt only.

Type I pneumococcal polysaccharide was not altered by treatment with 25 per cent dioxan for 4 hours at room temperature. Horse antiserum similarly treated gave delayed flocculation.

The author wishes to thank Professor J. R. Marrack for his advice and encouragement.

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INTRACEREBRAL INJECTIONS AND THE GROWTH OF VIRUSES IN THE MOUSE BRAIN

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THE intracerebral injection technique is one that has been widely used in animal virology, especially following the discovery by Theiler (1930) that yellow fever virus was pathogenic for mice when injected by this route. New viruses were thereby isolated and studied in a convenient laboratory animal. In spite of the fact that this technique has been in general use for the past 30 years, little is known of its mechanics, or of the distribution in the brain of the injected material.

When viruses which are injected intracerebrally to mice multiply in the mouse brain and produce a neurological type of illness, they are loosely referred to as "neurotropic" viruses. Less than 5 per cent of the cells in the brain, however, are neurones (Nurnberger and Gordon, 1957), and since the cells which support virus growth can now be identified in sections by the fluorescent antibody technique, a re-examination of the "neurotropism" of certain viruses has been made using this technique. Experiments on the mechanics of intracerebral injections, and the fate in the brain of the injected material, are also described.

In subsequent papers the adaptation of influenza and vaccinia viruses to mouse brain will be analysed by means of the fluorescent antibody technique.

MATERIALS AND METHODS

Mice.—Multicoloured outbred mice of the Walter and Eliza Hall Institute (Melbourne) strain were used.

Viruses.—The Moscow strain of ectromelia (mousepox) was used in the form of an infected liver suspension (Mims, 1959).

The Murray Valley Encephalitis (MVE) virus strain had received a very small number of mouse and egg passages since its isolation, and it was used in the form of an infected mouse brain suspension.

Titration methods.—Ectromelia titrations were made by the pock counting method as described in an earlier paper (Mims, 1959). Titres are expressed as pock forming units (pfu) per 0·05 ml. of tissue.

MVE virus titrations were made intracerebrally in adult mice and LD 50 titres calculated by the method of Reed and Muench (1938).

Fluorescence microscopy.—Fluorescent antibody staining by the direct method was used in most experiments. The more sensitive indirect method with rabbit antiserum was successfully used for ectromelia virus (Mims, 1959) to confirm results, but many rabbit antisera gave marked non-specific staining of the vascular bed of the brain which could not be prevented by thorough adsorption with either mouse brain or mouse liver powder. Most rabbit antisera also gave slight non-specific staining of nuclei in the brain parenchyma and, although this again could not be prevented by adsorption, the fluorescence it produced was not usually very bright.

Conjugates for the direct method were prepared from fluorescein isothiocyanate (Sylvana Chemical Co., Orange, New Jersey, U.S.A.) and γ globulins extracted from sera by Na_2SO_4 precipitation.

Vaccinial hyperimmune rabbit serum was used for ectromelia, and monkey immune serum (kindly supplied by Dr. S. G. Anderson) for MVE virus. Tissues were sectioned, stained, examined, and photographed as described in an earlier paper (Mims, 1959).

Routine histology.—Brains were fixed in formol saline and stained with haematoxylin and eosin.

RESULTS

Preliminary Experiments with Intracerebral Injections

The fate in the brain of injected material

It was shown by Cairns (1950), who used a phage label, that the greater part of an intracerebral inoculum spills over immediately into the blood stream. This was readily confirmed when it was observed that the liver of a mouse blackened a few seconds after an intracerebral injection of Indian ink. A study of the fate of the material which stayed in the brain was made by injecting 0·03 ml. of 10-fold diluted Indian ink intracerebrally in mice, and fixing, sectioning, and staining brain from 10 min. to 48 hr. later. Some unweaned mice were also used, and sections were then cut through the entire head, and through the spinal column. In all cases, the injected material was found to be distributed throughout the subarachnoid space, in many of the Virchow-Robin spaces surrounding blood vessels, and often, but not always in the ventricles (Figs. 1 and 2). In the spinal cord there was ink in the central canal and subarachnoid space, and it extended round the spinal nerve roots as they passed through the vertebral foramina. Indian ink was never observed in the parenchyma of the central nervous system except under the following three circumstances. First, in a few sections an Indian ink-containing fissure was seen, usually joined to a ventricle or subachnoid space and passing in the tissue plane between ganglia or fibre tracts ; such fissures were commoner when larger volumes were injected more rapidly. Second, in some sections the track of the injecting needle was seen, and this contained Indian ink. Third, in the brains removed after 1–2 days glial cells laden with Indian ink were often seen below the pia and ependyma, and it was thought probable that they had migrated into the parenchyma from the subarachnoid spaces or ventricles.

Thus, nearly all the intracerebrally injected material which stays in the brain is deposited throughout the cerebrospinal fluid (CSF) spaces (Fig. 13) and does not come into contact with parenchymatous tissue (Schaeffer and Muckenfuss, 1938). Small amounts may reach the parenchyma along the needle track, or when tissue planes are split by the injection pressure, or by being carried iuto the parenchyma by wandering macrophages (microglia).

The Pressure Used in Intracerebral Injections, and the Volume Injected

The important experimental work on the passage of material into the blood after its injection into the CSF has been done using carefully controlled physiological injection pressures and volumes. Massive overflows of injected material into the blood do not occur under these circumstances, and it is clear that anatomical barriers must be destroyed to make such overflows possible. A study of the pressures and volumes used in the usual mouse intracerebral injection technique was therefore made.

It was first noted that 0·03 ml., the volume traditionally injected, is a very large volume relative to the volume of the adult mouse brain, which is about

0·4 ml. An equivalent volume injected intracerebrally to a man would be 100 ml.

Injection pressures and rates of flow were investigated by injecting ether anaesthetized mice intracerebrally with saline from a long vertical tube. The tube (0.23 cm. diameter) was connected to a large (21 gauge) needle *via* a tap. The saline level was recorded at the start of each injection, which was continued for $\frac{1}{4}$ or $\frac{1}{2}$ min. One reading was taken from each mouse, and the averaged results from more than 200 mice are recorded in Fig. 14. It can be seen that there is no rapid flow of material into the mouse cranium until the injection pressure is about 150 cm. H_2O. The pressures exerted during routine intracerebral injections were found to be of the order of 200–300 cm. H_2O, and at such enormous pressures, 20–30 times the normal CSF pressure, the flow is quite rapid. There is no detect-

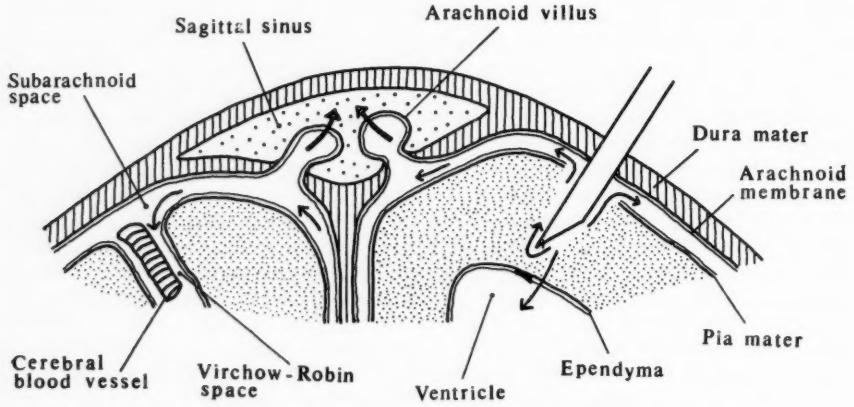


FIG. 13.—Diagram to show the fate of intracerebrally injected material.

able flow and a routine intracerebral injection could not be made, if the pressure is a mere five times the normal CSF pressure.

The skull can be thought of as a more or less rigid box enclosing incompressible material; for every 0·03 ml. which is forced in, 0·03 ml. has to be expelled. Minor adjustments can perhaps be made by the collapse of veins or the redistribution of CSF, but it is known that most of the injected material is in fact forced into the blood stream (see above). It would not be surprising if anatomical barriers were broken down by the use of high injection pressures. It seems likely that the first barriers to break down as the pressure rises, are the arachnoid villi. These relatively thin-walled structures form a barrier between CSF and blood (Fig. 13), and once they have been "blown open" there is a free flow of injected material into the blood. Indeed, from then on the injection becomes an intravenous one, and large volumes can be given. A volume of 0·5 ml. of saline has often been injected intracerebrally to mice and 5 ml. were injected into the one rabbit tested with no ill effects. If large volumes are injected too rapidly, additional anatomical structures are broken down, the inoculum may leak from the nose, and recovery is less probable.

In summary, the grossly unphysiological intracerebral injection methods in common use deposit some of the injected material in the CSF spaces and little or none in nervous tissue. The rest spills over into the blood stream. Injected viruses will necessarily be deposited in the same regions, and a study of the site of growth in the brain of intracerebrally injected viruses will now be reported, and the results considered from this point of view.

The Growth of Ectromelia (Mousepox) Virus Following Intracerebral Injection

General observations

Mice dying after extraneural injections of ectromelia virus did not have brain titres of more than $10^{3.0}$ pfu/0·05 ml. brain, even when the brain was traumatized

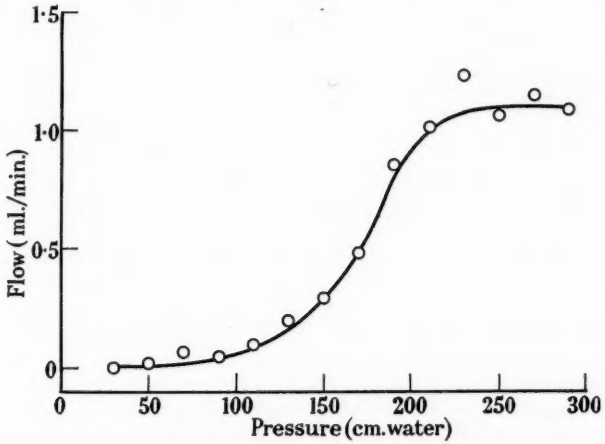


FIG. 14.—The relation between the rate of flow of intracerebrally injected saline and the injection pressure.

at the time of injection. Such titres are attributable to the virus present in the blood in the brain. When, however, large doses of virus are injected intracerebrally into mice, there is growth of virus in the brain, and titres of 10^6 pfu/0·05 ml. brain are common. Death, which occurs 2–3 days after large intracerebral injections, is preceded by a neurological type of illness. Sick mice tremble, have ruffled fur, are hunched, and may die during the course of convulsions. Convulsions can often be induced by tail twirling and sometimes by merely hanging the mouse upside down. Paralysis never occurs. There is detectable growth of virus in the brain when more than about 10^3 pfu are injected intracerebrally, in that titres of more than 10^4 pfu/0·05 ml. brain are reached by the time of death, and the neurological type of illness may occur.

Most of the intracerebrally injected material is forced into the blood stream, so that there is growth of virus in the viscera, just as there is after intravenous injections. Unless they die in a convulsion mice probably die as a result of this

visceral growth of virus rather than as a direct result of growth in the brain, for survival times were the same and equally high liver titres were attained after large doses of virus, whether given intravenously or intracerebrally. It was also shown that the intracerebral injection route is as sensitive as the intravenous one as a test for virus, because a given material gave the same titres (both ID 50 and LD 50) when titrated intracerebrally and intravenously.

An attempt was made to adapt ectromelia to the mouse brain by making 10 brain passages, using large doses at each passage. The brain titres and survival times of injected mice, however, were unchanged. Jahn (1939) also found no increase in the neurotropism of ectromelia virus after 20 brain passages in mice.

Growth curve experiment

$10^{7.2}$ pfu of ectromelia virus were injected intracerebrally (0·03 ml.) in many mice, and at intervals afterwards 3 mice were killed with ether, their brains pooled, suspended in 6 ml. gelatine saline, and titrated *in ovo*. The time of onset of sick-

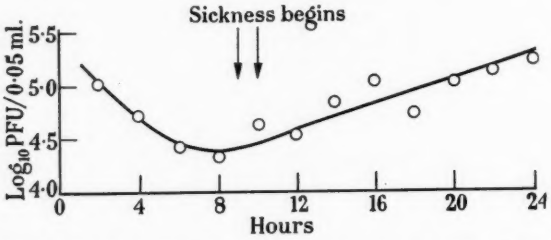


FIG. 15.—The growth of intracerebrally injected ectromelia virus in the brain of mice.

ness was carefully observed. There was a preliminary disappearance of virus in the brain, followed by a gradual rise in titre which began after about 8 hr. (Fig. 15). All mice were well until the ninth hour, when some of them were slightly sick, and most were sick by 12 hr. ; the first mice died after 2 days. Sickness begins much later after large intravenous injections of virus, and is not detectable until at least 24 hr., however much virus is injected (Mims, 1959). This experiment shows that ectromelia virus grows in the mouse brain producing sickness soon after growth first begins.

The site of growth of virus in the brain

Brains from mice moribund or very sick 2–3 days after large intracerebral injections of virus were quick-frozen and sections examined by the fluorescent antibody technique. Specific fluorescence was limited to cells lining the CSF spaces, that is, to cells lining the ventricles, subarachnoid space, and Virchow-Robin spaces (Figs. 3–9). The few fluorescent cells seen just below the pia and ependyma were probably glial cells which had migrated into the parenchyma like the Indian ink-laden cells described above. Larger numbers of glial cells were sometimes seen in the parenchyma adjacent to the third ventricle. There was no fluorescence lining the needle tracks even when the intracerebral injection was made with multiple insertions of the needle. Brains examined 7 and 17 hr. after

the injection of virus showed a fainter specific fluorescence, which was, however of similar distribution.

Thus intracerebrally injected ectromelia virus grows mainly in the meninges and ependyma, and not in neural tissue proper. This finding was confirmed in a crude way when brains from moribund infected mice were frozen, and segments cut from meninges and parenchyma with a scalpel. When these segments were suspended in gelatine saline and titrated, it was found that nearly all the virus was in the meningeal material.

Again, the type of sickness in intracerebrally infected mice is meningitic rather than encephalomyelitic, and brains show the pathological changes of meningitis and ependymitis on routine histological examination.

In other fluorescent antibody experiments it was shown that there was the same distribution of antigen in the brains of mice very sick following the intracerebral injection of rabbitpox virus.

The Growth of MVE Virus in Mouse Brain

Since ectromelia had been shown to be meningotropic rather than neurotropic, MVE virus, which might be expected to behave in a more strictly neurotropic fashion, was also studied.

Mice were injected intracerebrally with 10^7 LD 50 of MVE virus and brains were taken at the time of sickness 4–5 days later, sectioned, and examined by the fluorescent antibody technique. In these brains there was no meningeal or ependymal fluorescence, but there was a bright specific fluorescence of many parenchymal cells (Plate, Figs. 10–12). Some of these cells were almost certainly neurones, although even after haematoxylin and eosin staining it was not possible to say with complete certainty that they were not neuroglia.

Intracerebrally injected MVE virus is mechanically distributed through the CSF spaces, as is Indian ink, and in an attempt to find out how it reaches susceptible parenchymal cells, brains were examined 24 and 48 hr. after large intracerebral injections of virus. No antigen was seen at 24 hr., and small parenchymatous foci were established by 48 hr. In neither case was there antigen in the cells lining the meninges, ependyma, or Virchow-Robin spaces. Another brain was examined 24 hr. after a large amount of virus had been given intracerebrally through five separate injection sites. There was no antigen in the cells lining the needle tracks.

MVE virus thus grows in parenchymal cells after intracerebral injection into mice. Virus probably does not reach susceptible cells by way of the injecting needle track, nor by growing through the ependyma or meninges. Perhaps it is carried through to the parenchyma by wandering macrophages, as was ectromelia virus.

DISCUSSION

It has been shown that high pressures are needed to inject mice intracerebrally by the usual method. When Indian ink is injected it overflows into the blood stream and is also deposited in the spaces occupied by the CSF. Viruses injected in the same way will necessarily be deposited in the same spaces, and the cells lining these spaces will be the ones immediately available for viruses to grow in. Ectromelia (mousepox) virus, rabbitpox virus and probably many other pox viruses, grow in these cells, producing a meningitis and ependymitis which is

detectable histologically and results in a meningitic type of illness. There is, however, no growth in the brain parenchyma. Buddingh (1938) showed that intracerebrally injected fowlpox virus produced lesions in the meninges, perivascular spaces and ependyma of chicks, with no primary damage to nervous tissue.

It was Levaditi and Nicolau (1923) who first placed certain strains of vaccinia virus in the general category of neurotropic viruses, although Hurst and Fairbrother (1930) reported that monkeys and rabbits injected intracerebrally with neurovaccinia showed a mainly meningeal histological reaction. Hurst and Fairbrother stated that " the virus of vaccinia is not neurotropic in the same sense as that of poliomyelitis ". This conclusion is amply confirmed by the fluorescent antibody studies reported here.

The fluorescent antibody evidence shows that MVE virus grows in the cells in the parenchyma of the brain but that it does not grow in the cells lining the CSF space. The intracerebrally injected virus could cross the CSF-brain barrier and reach parenchymal cells by any of the three routes referred to above for the Indian ink experiments, or by " growing through " ependymal or pial cells as in the case of the NWS strain of influenza virus (Mims, unpublished). There is, however, no detectable growth of virus in the ependyma or meninges, nor in the injecting needle track. The needle track also seems less likely to be important because poliovirus, when carefully injected into the subarachnoid spaces of monkeys with no damage to the underlying tissues, still grows in neurones to produce the typical disease (Hurst, 1932). Virus does not reach the parenchyma along tissue planes split open by the injected material because it was found that there was growth as usual in the brain when a virus-contaminated needle by itself was inserted intracerebrally. This leaves open the possibility that migrating macrophages carry MVE virus across the CSF-brain barrier.

EXPLANATION OF PLATES

FIG. 1.—Haematoxylin and eosin stained section of the brain of a mouse 10 min. after the intracerebral injection of Indian ink. The ink is restricted to the subarachnoid space at the surface of the brain. (× 33).

FIG. 2.—Similar section containing the hippocampus and neighbouring structures with the Indian ink again restricted to the subarachnoid space. (× 13).

FIG. 3.—Fluorescein labelled ectromelia antigen in mouse brain.

FIG. 4.—Same field stained with haematoxylin and eosin, to show the extensive areas of antigen-free parenchyma, and the localization of antigen to the subarachnoid space of a nearby sulcus. (× 10).

FIG. 5.—Fluorescein labelled ectromelia antigen in mouse brain.

FIG. 6.—The same field stained with haematoxylin and eosin, showing that antigen is restricted to the subarachnoid space of the meninges, although a few fluorescent cells are seen in the adjacent parenchyma. This mouse received an intracerebral injection of Indian ink shortly after the virus injection. Blood covers the meninges, and there is a large vein in the subarachnoid space. (× 60).

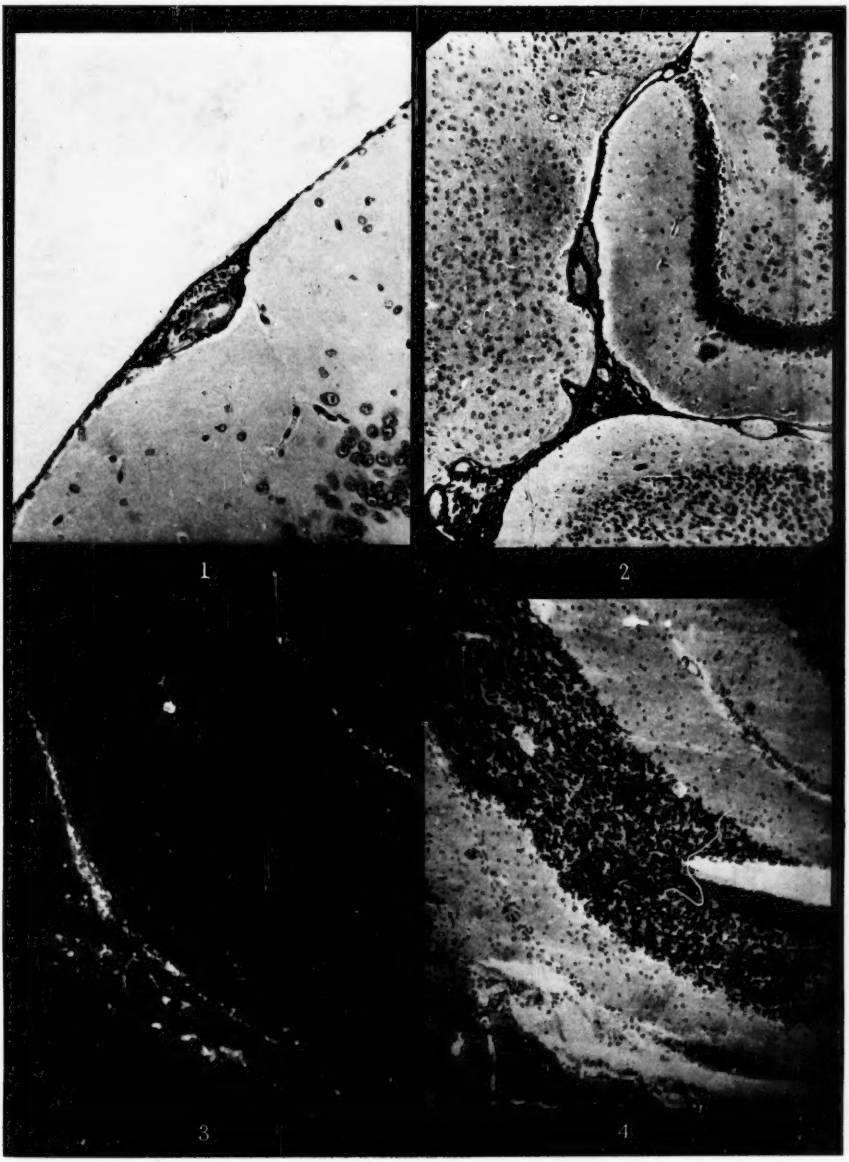
FIG. 7.—Fluorescein labelled ectromelia antigen in mouse brain.

FIG. 8.—The same field stained with haematoxylin and eosin. Antigen is present only in the ependymal cells lining the ventricle. (× 150).

FIG. 9.—Fluorescein labelled ectromelia antigen in mouse brain, present in the Virchow-Robin spaces surrounding blood vessels. (× 80).

FIG. 10.—Fluorescein labelled MVE antigen in mouse brain. The meninges contain no antigen, although a small focus of antigen-containing cells is present in the neighbouring parenchyma. (× 40).

FIGS. 11 and 12.—Fluorescein labelled MVE antigen in mouse brain. Parenchymatous cells contain antigen which is limited to the cytoplasm. (× 700).



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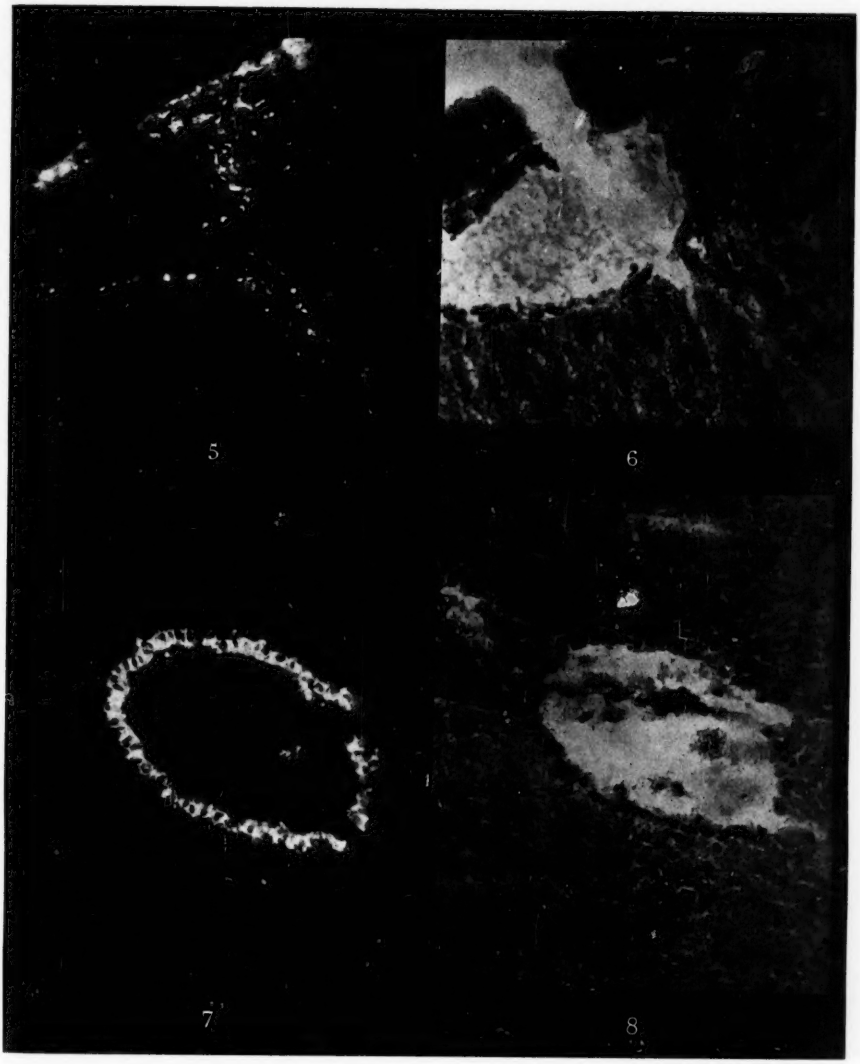
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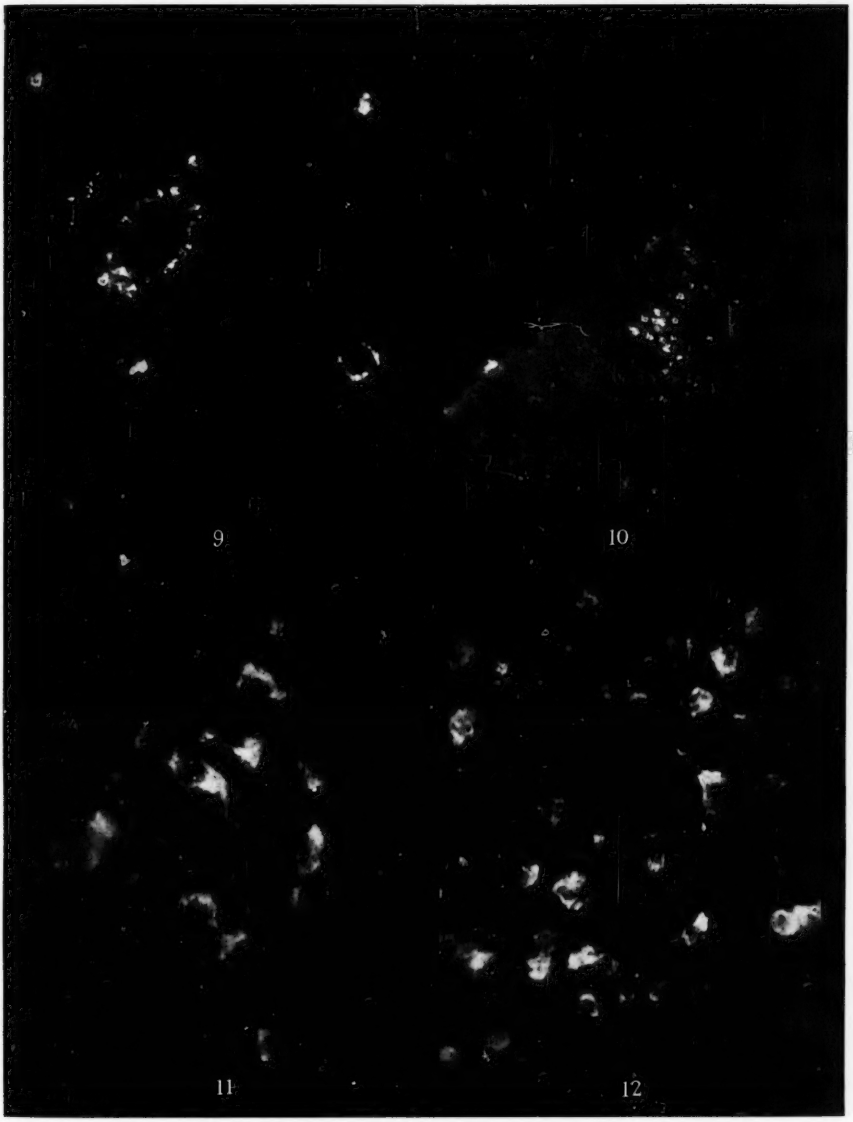

Mims.

It can be noted that viruses which grow in the continuous sheet of cells lining the CSF space, and which may kill the mouse when a large proportion of these cells have been involved, can be lethal within a short time. Viruses which grow in neurones or neuroglia, however, have to spread through a complex three-dimensional cell system after each cycle of growth. The ground substance of the brain (neuropil), far from being an extracellular fluid permitting the free diffusion of virus through it, is in fact a complex system of cytoplasmic processes from other cells (Palay, 1958). Foci enlarge irregularly as virus spreads from infected cells to those in contact with them or with their processes, until certain regions of the brain are lethally damaged, for it is probably growth in certain important regions of the brain, rather than the total amount of growth, which proves fatal. Thus, although a large intracerebral injection of rabbitpox virus kills mice in 2 days, the largest dose of MVE virus which can be given does not kill until the fourth day.

The fluorescent antibody technique was also used by Noyes (1955) and Lebrun (1956) to study the growth of viruses in the mouse brain. Noyes showed that West Nile virus, closely related to MVE virus, grew in neurones, while Lebrun concluded that Herpes Simplex virus grew in glial and ependymal cells, but not in neurones.

SUMMARY

Very high pressures are needed to inject mice intracerebrally, and large volumes are usually injected. Some of the injected material is deposited in the CSF spaces in the brain, but the high injection pressure breaks down the CSF-blood barrier, so that the rest spills over into the blood.

The fluorescent antibody technique has been used to identify the cells in the mouse brain which support the growth of certain intracerebrally injected viruses.

Intracerebrally injected ectromelia and rabbitpox viruses grow in the cells lining the cerebrospinal fluid spaces to produce meningitis and ependymitis. There is little or no growth in the brain parenchyma.

Intracerebrally injected Murray Valley Encephalitis virus grows in neurones or neuroglia, and not in cells lining the CSF spaces. Its passage across the CSF-brain barrier has been investigated.

It is a pleasure to acknowledge the competent histological assistance of John Benge.

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THE RELATIONSHIP BETWEEN INTERMITTENT HYPOTENSION AND THE PREVENTION BY HYDRALAZINE OF ACUTE VASCULAR DISEASE IN RATS WITH STEROID HYPERTENSION

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THE rapid development of hypertension in albino rats given salt and cortexone (deoxycortone acetate) is usually accompanied by the appearance of widespread acute vascular disease (Selye, Hall, and Rowley, 1943 ; Masson, Hazard, Corcoran and Page, 1950). The disease is focal in distribution and involves both small and medium-size arteries, as an arteritis, and arterioles in which the presence of subendothelial material with the tinctorial characteristics of fibrinoid accompanies a slight adventitial inflammatory reaction. In both types of vessel the form and evolution of these changes closely resemble those found in rats with renal (Wilson and Byrom, 1939 ; Loomis, 1946), pituitary (Masson *et al.*, 1950) or adrenal-regeneration (Skelton, 1955, *b*) hypertension.

Many investigators assume that these forms of vascular disease in the rat, arterial and arteriolar, are part of a species-specific response to hypertension (Masson *et al.*, 1950, Koletsky, 1955) although sufficient direct evidence of the validity of this interpretation, obtained from comprehensive comparative morphological studies, is not yet available. The animal lesions are therefore regarded as the experimental counterpart of the fibrinoid arteriolar change pathognomonic of the malignant phase of hypertension in the human (Fahr, 1919 ; Heptinstall, 1953). Both the human and the rat vascular disease are thought to be the results of a critical rise in blood pressure (Pickering, 1955). This view receives strong but indirect support from the demonstration that in experimental hypertension of renal origin in the rat vascular damage does not develop in the kidney to the artery of which a constricting clip has been applied (Wilson and Byrom, 1939 ; Floyer, 1951). By contrast, Bali and Goldblatt (1954) submit that while the necrotizing arteriolar lesions in renal hypertensive rats are dependent upon at least two factors including both hypertension and functional renal failure, the necrotizing arteritis is non-specific and independent of blood pressure.

No direct evidence is available relating intra-arterial and intra-arteriolar pressure changes to the evolution in the same vessel of mural damage in rats with experimental hypertension. The only satisfactory approach to this type of observation is provided by the work of Byrom and Dodson (1948), of Schaffenburg and Goldblatt (1957), and of Wolfgarten and Magarey (1959). Byrom and Dodson (1948) demonstrated that acute vascular disease was produced in normotensive rats by the introduction under pressure of fluid into large arteries remote from those subsequently found to be damaged. Their experiments were repeated by Schaffenburg and Goldblatt (1957) without confirmation. However, comparable work was described by Wolfgarten and Magarey (1959) who claimed to have caused

acute arterial damage by this means. Their descriptions and photographs, nevertheless, do not provide acceptable evidence that the arterial lesions which they produced were identical with those found in the commonly studied forms of hypertension in the rat. They were apparently not successful in reproducing arteriolar lesions.

In view of the complete lack of knowledge of the effects produced by such high central intravascular pressures on vascular tone, pressure and blood flow in small arteries and in arterioles of similar size to those damaged in experimental hypertension, considerable caution must be exercised in deducing from any part of the evidence presented in these three papers that the acute vascular injuries of experimental hypertension in the rat are the result of direct pressure changes. Equally, in the absence of direct human demonstration care must be exerted in reaching similar conclusions in regard to the vascular disease of the malignant phase of human hypertension. The need for direct evidence in this respect is not admitted by many authorities, perhaps with some justification in view of the strong indirect evidence available (Pickering, 1955).

An impetus was given to the search for the cause of the acute vascular disease in hypertensive rats by the demonstration that it was possible to delay, modify or suppress such changes by prolonged treatment with hypotensive drugs (Masson, McCormack, Dustan and Corcoran, 1958). In these experiments the hypotensive drug hydralazine (1-hydrazinophthalazine) was given orally to rats in amounts shown to be sufficient to prevent a significant rise in blood pressure on a regime known to cause severe renal hypertension. These results indicated that apparently constant control of systolic blood pressure exerted in this way was associated with the virtual absence of acute renal vascular disease, that vascular disease developed if treatment with hydralazine was stopped, and that a period of uncontrolled renal hypertension led to the appearance of acute vascular lesions which responded to delayed treatment by healing. Both arterial and arteriolar changes behaved similarly. These experiments therefore confirmed that apparent control of systolic renal hypertension in the rat was accompanied by preservation of the integrity of the walls of small arteries and arterioles. From this evidence the deduction was made that the acute vascular lesions in animals with renal hypertension were the consequence of the raised blood pressure, an opinion in support of which no satisfactory direct evidence was offered.

During recent work on the toxicity of hydralazine (Gardner 1957, 1958), and for reasons not related to changes in blood pressure, it became customary to give this drug in large amounts by single daily intramuscular injection, for prolonged periods, to rats with steroid hypertension. In the course of this work it was noticed that treatment was exerting an unexpectedly severe influence on the evolution of the characteristic vascular changes caused by salt and cortexone (Gardner, 1959). Further experiments were therefore undertaken to extend these observations and to define in more detail the circumstances under which hydralazine exerted this effect. The results of the first of these experiments are presented in this paper.

MATERIALS AND METHODS

Animals.—Male and female Wistar (groups 5 and 6) and Sprague-Dawley (groups 1, 2, 3, and 4) rats were maintained on a diet containing all known essential nutritional factors. The mean weight of these rats varied from 123 to 190 g. The animals were divided at random into paired groups. Three paired groups were studied consecutively.

Cortexone (deoxycortone acetate) was used in the form of compressed tablets for subcutaneous implantation, or as a 1·25 per cent aqueous suspension with tragacanth. The reactivity of the tragacanth was excluded by the daily injection of this agent alone into a distinct group of animals for a period of 10 weeks.

Salt was given as a 1 per cent aqueous solution of analytical grade sodium chloride in tap water.

Hydralazine (Apresoline-Ciba) was used as a sterile 2 per cent solution.

Systolic blood pressures were recorded weekly by tail plethysmograph, with (groups 5 and 6) or without (groups 1, 2, 3, 4) light ether anaesthesia (Dodson and Mackaness, 1957). The use of anaesthesia did not significantly change the effects of hydralazine while the influence of ether as a hypotensive agent was excluded by its use in an independent experiment, not described here, in which an unreactive chemical was substituted for hydralazine in a further control group of animals.

Diastolic blood pressures were measured under pentobarbitone anaesthesia using a carotid cannula connected to a pressure transducer. The pulse waves were visualised on an oscilloscope and transcribed to paper with a direct writing recorder.

Histological material was fixed in corrosive formol or in neutral formol saline; paraffin sections were stained routinely with haematoxylin and eosin and by the Picro-Mallory method, while other staining techniques were used when necessary.

Procedure

The animals of each group were weighed, and the systolic blood pressures recorded. After not less than 1 week of observation, to exclude illness or infection, left nephrectomy was performed under ether or pentobarbitone anaesthesia. At the same time implants of 35 mg. of cortexone (groups 1 and 2) or 70 mg. of cortexone (groups 3 and 4) were inserted subcutaneously in the posterior cervical region by separate incision. Injections of 2·5 mg. of cortexone suspension were given daily to the animals of groups 5 and 6. Within 4–7 days after recovery from operation, 1 per cent salt was substituted for the drinking water. At

TABLE I.—*Composition of Animal Groups*

Group	Males	Females	Total	Mean initial weight (g.)	Mean initial B.P. mm./Hg.	Cortexone (deoxycortone)	Maximum dose of hydralazine (mg./kg.)	Duration of treatment (weeks)
1	12	0	24	188	117	35 mg. subcutaneous implant	—	4
2	12	0		188	106		Up to 25	
3	7	0	14	190	110	70 mg. subcutaneous implant	—	8
4	7	0		190	111		Up to 35	
5	4	4	16	116	107	2·5 mg. of suspension daily	—	12
6	4	4		130	100		Up to 50	

the same time the daily injection of hydralazine was begun. The initial dose of the drug was 1 mg. in each instance, but this amount was quickly and progressively increased. After 12 weeks some surviving animals of group 3 were receiving as much as 8 mg. daily. Animals of group 2 were treated in this manner for 4 weeks, those of group 4 for 8 weeks and those of group 6 for 12 weeks. The injections were given each day without exception.

At the close of the respective periods of treatment animals were killed by bleeding under anaesthesia. Representative parts of the principal organs were placed in neutral formol saline (groups 1–4) or in corrosive formol (groups 5 and 6).

At intervals during the study of each experimental group selected animals were subjected to more detailed investigation in order to determine the diurnal variation in systolic blood pressure in response to treatment. Towards the end of the experiment the diastolic blood pressure of a smaller number of these animals was measured, and the effects of the daily injection of hydralazine estimated by direct manometry.

RESULTS

The general condition and well-being of the animals

Animals injected once daily with hydralazine remained in better health than those not so treated. Many gained weight more rapidly than control animals. This effect was particularly obvious in groups treated for 8 or for 12 weeks.

Systolic blood pressure

During the preliminary experiments on the toxicity of hydralazine systolic blood pressures were recorded 4–6 hr after the daily injection of hydralazine. The data obtained by this method suggested that the mean systolic blood pressure was maintained within the limits of normality (Fig. 1). More detailed analysis

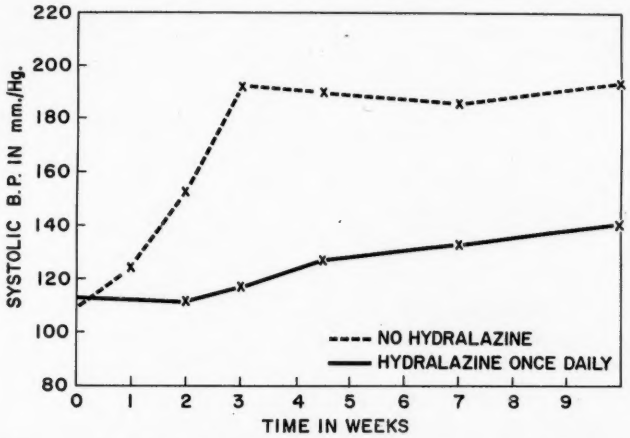


FIG. 1.—The influence of once-daily injections of hydralazine on the mean systolic blood pressures of albino rats with steroid hypertension. (Blood pressures measured 4–6 hr. after each injection).

quickly showed that this procedure was misleading and that following each daily injection the systolic blood pressures underwent a severe fluctuation (Fig. 2). Immediately after each injection the pressure fell abruptly, reaching a minimum within 1½–3 hr., rising again more slowly and resuming the pre-injection (hypertensive) level within 18–24 hr. Among the animals in each group there was considerable individual variation in response, because of the injection of equal doses of hydralazine into animals of varying body weight (Fig. 3).

Diastolic pressure

In view of the wide variations in systolic blood pressure, and because of the suspected inaccuracy of the plethysmographic method of measurement at levels below 80–100 mm. Hg., it was felt that an attempt should be made to confirm the extent and severity of representative blood pressure responses by direct manometry. With a sensitive recording device the rapid pulse wave of the hyper-

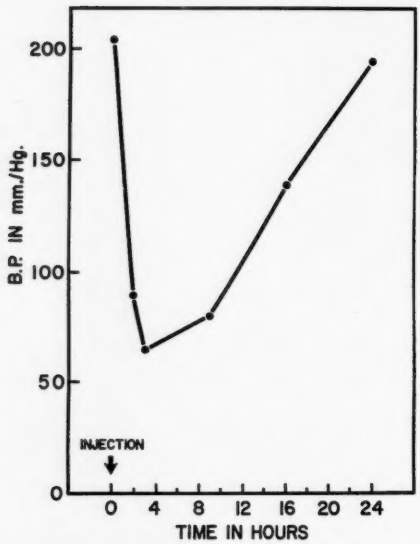


FIG. 2.—The change in mean systolic blood pressure in a group of 12 rats with steroid hypertension during the 24 hr. following a single large intramuscular injection of 6 mg. hydralazine.

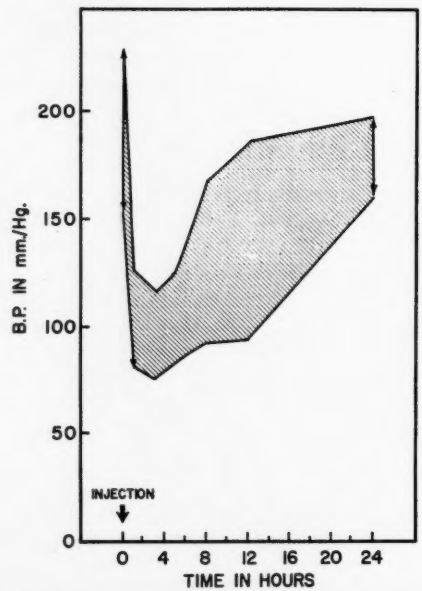


FIG. 3.—The range of systolic blood pressure readings recorded among a group of 12 rats with steroid hypertension during the 24 hr. following a single intramuscular injection of 7 mg. hydralazine.

tensive rat could be analysed sufficiently to measure both diastolic and systolic pressures (Fig. 4). Readings made in this manner showed that both systolic and diastolic pressures began to fall within 3 min. after the usual intramuscular injection of hydralazine, and that the fall continued for periods of ½–1½ hr. before reaching a plateau (Fig. 5). The low pressure levels were maintained for 1–2 hr. before a rise began, and the levels present before injection were resumed in less than 24 hr. In each example measured in this way diastolic pressure fell in parallel with systolic. In other preliminary experiments in which the drug was injected intravenously the very severe and abrupt response which followed appeared to affect systolic pressure more quickly than diastolic (Fig. 6).

For reasons which are not at the moment clear, but which have been commented upon by other observers (Caster, Lentz, Poncelet and Armstrong, 1956) it proved difficult to make a direct quantitative comparison between pressure levels recorded directly and those recorded indirectly. Direct carotid manometry appeared to yield higher systolic and diastolic pressure in both normal and hypertensive rats irrespective of whether blood flow through one or both carotid arteries was disturbed. Such pressures were significantly more sensitive to hydralazine than were those recorded indirectly. Further, direct manometry not only confirmed the form of response to hydralazine already detected by indirect manometry but revealed more rapid and more extensive effects. For the present purpose the quantitative difference between the results obtained by the individual methods of manometry was therefore disregarded. Argument based on the indirect measurement appeared to gain significance when contrasted with the conclusions drawn from direct manometry.

Weight of organs

The mean weight of the kidney from animals treated for only 4 weeks (group 2) expressed as a percentage of terminal body weight was found to be less than that of the corresponding control group (group 1) not given hydralazine. In animals treated for longer periods of 8 or 12 weeks (groups 4 and 6) the kidneys were appreciably heavier than those of the control groups (3 and 5).

In considering the effect of treatment in different groups on the mean heart weight expressed as a percentage of terminal body weight, it is necessary to

TABLE II.—*Results : Effect of Treatment on Mean Body Weight and Mean Organ Weights*

(Weights expressed in grams)

Group	Mean body weight at death	Mean weight change	Right kidney	Heart	Adrenals (combined)
1	225	+37	1·99 (0·88%)	0·91 (0·41%)	0·046 (0·021%)
2	198	+10	1.53 (0·78%)	0.85 (0·43%)	0.043 (0·022%)
3	273	+83	2·37 (0·86%)	0·92 (0·34%)	—
4	312	+122	3.01 (0·96%)	1.11 (0·35%)	—
5	156	+40	1·40 (0·90%)	1·25 (0·80%)	—
6	228	+98	3·02 (1·32%)	2·24 (0·98%)	—

emphasize that the method for weighing the hearts of animals in groups 5 and 6 differed from that used in weighing the hearts of animals in groups 1–4. With this proviso it remained clear that in most instances treatment with hydralazine did not significantly alter the mean relative heart weight, while in the groups treated for the longest period (groups 5 and 6) the injection of hydralazine led to an apparent increase in the mean relative weight of the hearts.

The terminal weight of the adrenals was determined in groups 1 and 2 and expressed in terms of total body weight. Treatment with hydralazine did not cause a significant change in the mean relative weight of the adrenals.

Histology

In control animals with steroid hypertension, vascular lesions were usually widespread within 4 weeks of beginning the raised sodium intake. The vascular changes were acute, and were of the fibrinoid arteriolar type characteristic of severe hypertension in the rat (Wilson and Byrom, 1939) and of the arteritic type whose origin remains in dispute (Masson *et al.*, 1950 ; Bali and Goldblatt, 1954 ; Koletsky, 1955). Both lesions are widely known and have been described repeatedly on previous occasions (Masson *et al.*, 1950 ; Skelton, 1955, *a*). Representative examples are shown in Figs. 7–10. The distribution of fibrinoid arteriolar change in a group observed for 4 weeks is shown in Table III. Mesentery, pancreas,

TABLE III.—*Group I : Distribution of Vascular Lesions in Surviving Animals*

Rat No.	Kidney	Adrenal	Heart	Lung	Mesentery	Pancreas	Lymph nodes	Liver	Spleen
97	+	–	+	–	+	+	0	+	0
99	+	–	0	0	+	+	0	0	–
100	+	0	+	0	+	+	–	0	0
101	+	–	0	0	+	+	0	0	0
102	+	—	0	0	+	–	–	0	0
103	+	–	0	0	+	+	0	+	–
104	+	–	0	+	+	0	0	+	0
105	+	0	+	0	+	0	+	0	0
Total	8	0	3	1	8	5	1	3	0

+ = Acute vascular disease. 0 = No vascular disease. – = Not examined.

EXPLATION OF PLATES.

FIG. 4.—Pulse wave of 190 g. rat with steroid hypertension shortly after the intramuscular injection of 8 mg. hydralazine. Pulse rate 460/min.

FIG. 5.—Fall in systolic and in diastolic blood pressures of 190 g. rat with steroid hypertension following the intramuscular injection of 8 mg. hydralazine. The point of injection lay immediately to the left of tht part of the tracing shown in this figure.

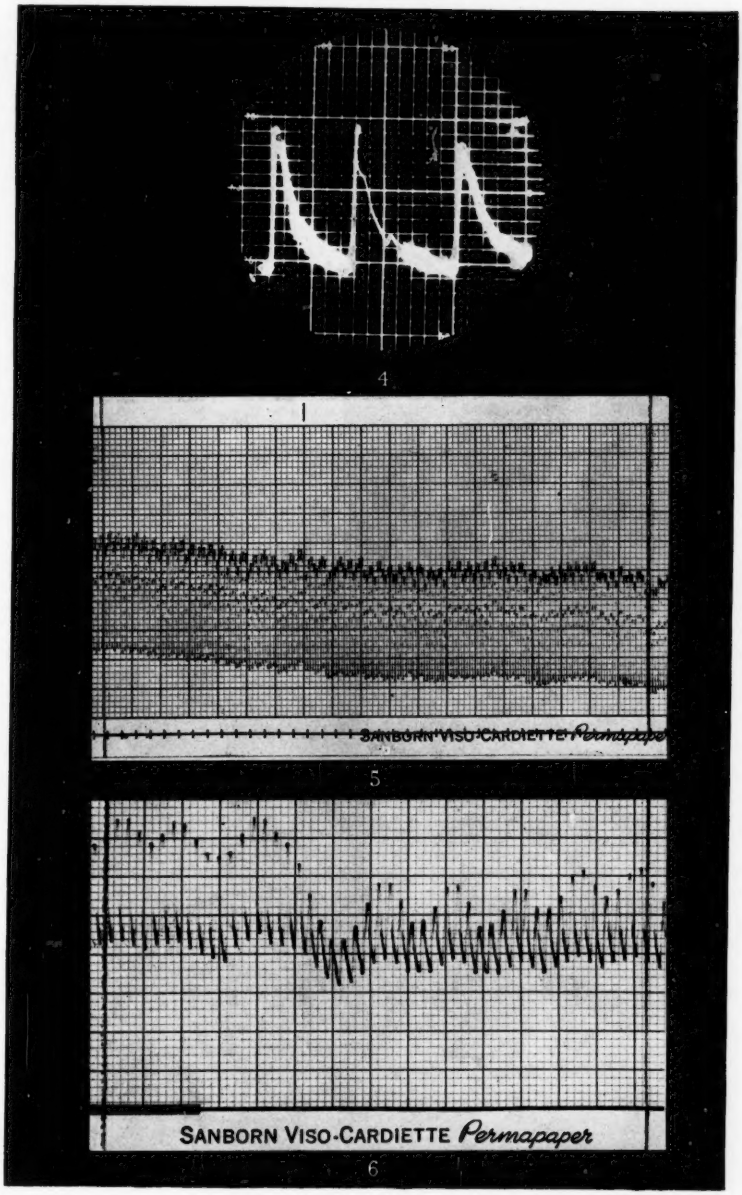
FIG. 6.—For comparison with Fig. 5, the fall in systolic and in diastolic blood pressures of a 260 g. rat with steroid hypertension following the intravenous injection of 0·5 mg. hydralazine.

FIG. 7.—Right kidney from control animal of Group 1. Material with the staining characteristics of fibrinoid occupies much of the enlarged glomerulus and forms part of the acute arteriolar reaction. There is the usual tubular dilatation. Haematoxylin and Eosin × 133.

FIG. 8.—Arteriolitis, with the presence of much subintimal fibrinoid, in a mesenteric lymph node from the same animal. H. and E. × 133.

FIG. 9.—A focus of ventricular myocardial inflammation, with arteriolar fibrinoid and perivascular fibrosis, in an animal of Group 1. H. and E. × 133.

FIG. 10.—Necrotizing arteritis of a medium-size mesenteric artery. The inflammatory reaction is severe. Subendothelial hyaline material is present. H. and E. × 53.



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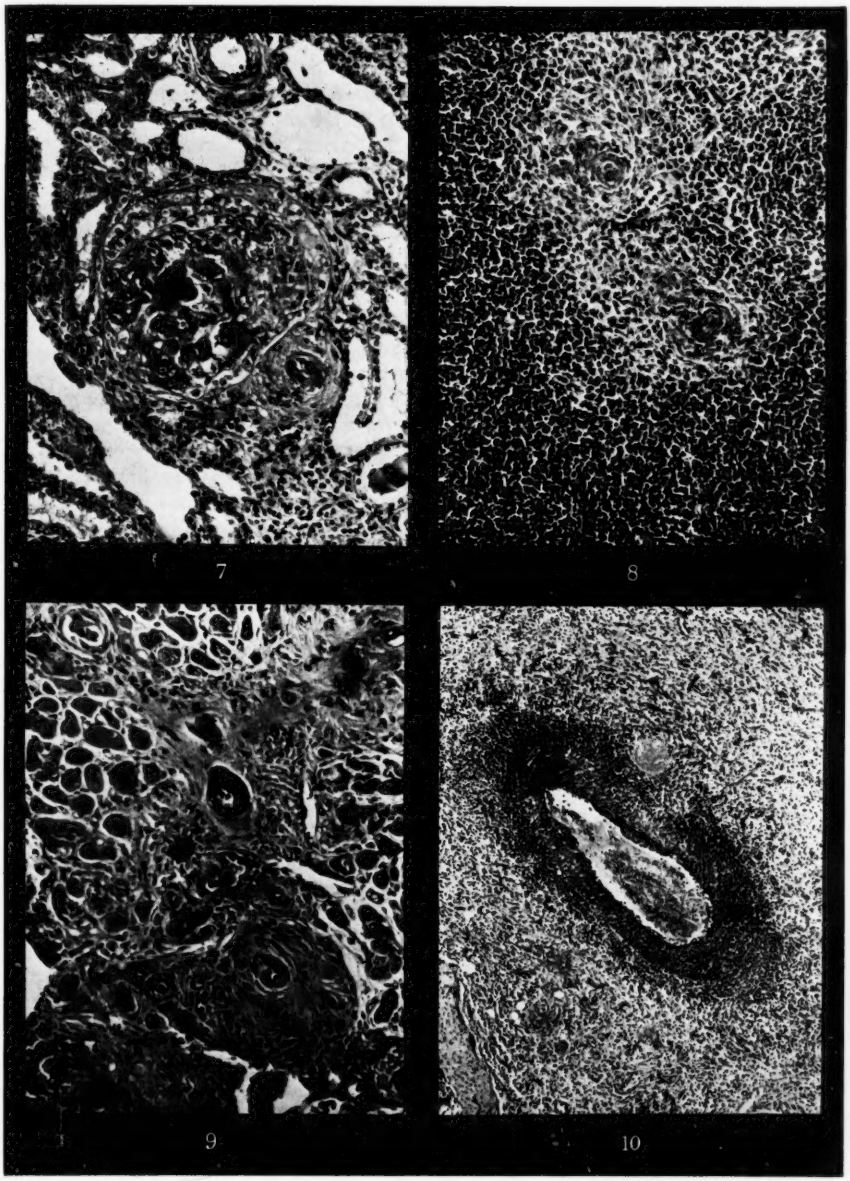

Gardner.

omentum and kidney were implicated most frequently, but acute arteriolar fibrinoid change was common in heart, lymph nodes, liver, adrenals and other organs. Acute arteritis of the so-called necrotizing variety was also common, and was found most often in regions related to the sites of previous surgical intervention. The lesions were not limited to these places however and were also commonly present in the mesenteric border of the gut, in the kidney and in other sites with increasing frequency as the period of observation lengthened. In rare instances, and for no apparent reason, an animal failed to develop vascular disease within the period of observation. This failure occurred only when subcutaneous hormone implants were used and was not noted in groups given daily subcutaneous injections of cortexone.

In animals given hydralazine, fibrinoid arteriolar lesions were few. Very occasionally an isolated arteriole appeared damaged. This was interpreted as individual biological variation in response, possibly associated with varying rates of growth in the face of constant doses of hydralazine. Many small and medium-size arteries in the mesentery, omentum and pancreas were surrounded by aggregates of inflammatory cells. The majority of these cells were histiocytes, apparently containing the residual detritus always found in the retroperitoneal space following the operation of unilateral nephrectomy and associated with the presence of foreign suture material at the site of renal arterial ligation. In no instance however were the walls of these vessels infiltrated by inflammatory cells, and in none was subendothelial eosinophilic hyaline or fibrinoid present.

Treatment with intermittent hydralazine therefore appeared to offer virtually complete suppression of both acute arteriolar fibrinoid and of acute necrotizing arteritis in rats with steroid hypertension for periods of up to 3 months while leading to long-continued profound repetitive fluctuations in systolic and in diastolic blood pressures.

DISCUSSION

Hydralazine is known to lower the systemic pressure of rats with experimental steroid hypertension (Gaunt, Antonchak, Miller and Renzi, 1955), with renal hypertension (Gross, 1955), and with adrenal-regeneration hypertension (Chart, Ulsamer, Quinn, Howie, Sullivan and Gaunt, 1957). The mechanism by which the drug exerts its effect is not fully understood but it may include central (Schmitt and Gicquel, 1955), renal (Hartman and Flagg, 1956) and peripheral vascular components (Schmid and Kellner, 1953). Tolerance to the drug is quickly acquired and large amounts can be given to rats without evident harm. When the drug is administered by single daily injections, as in the present experiment, an abrupt fall in systolic and in diastolic blood pressure is succeeded, in the hypertensive animal, by a less rapid rise.

It was with the express purpose of avoiding such fluctuations in blood pressure that Masson *et al* (1958) abandoned the administration of hydralazine to rats by stomach tube, preferring to substitute a hydralazine solution for the drinking water. This enabled a constant influence to be exerted on the blood pressure level, maintaining systolic pressure apparently within normal limits. No mention was made of the possibility that the nocturnal feeding and drinking habits of the rat may have disturbed this constant state and nocturnal blood pressure records were not quoted. Nevertheless treatment was successful in preventing the appearance of acute vascular disease.

In the present experiment treatment of rats with large single daily intramuscular injections of hydralazine resulted not only in abrupt daily falls in blood pressure but in rapid subsequent rises to preinjection levels. It appears reasonable to compare these rises with those which various authors hold responsible for the development in severe hypertension of acute damage to small arteries and arterioles (Pickering, 1955). However this comparison may only be made with care since it is not certain that the small vessels during recovery from a single injection of hydralazine are in the same morphological or physiological state as are those during rapidly progressive acute experimental hypertension. Direct visual observation of exposed mesenteric vessels following the injection of hydralazine into rats with steroid hypertension has not yet provided unequivocal evidence on this point, possibly because the small vessels studied were not those which determine systemic blood pressure control (unpublished personal observations).

If the vascular lesions of other forms of experimental hypertension in the rat are accepted as similar to those of steroid hypertension, and as far as the arterial and arteriolar disease is concerned this appears to be the case, the present experiment suggests the generalization that complete control of absolute blood pressure levels in the systemic circulation is not essential for the prevention of acute hypertensive vascular disease. Because of the different circumstances under which the experiments were conducted the present results do not conflict with those of Masson *et al.* (1958), but they suggest that the interpretation placed upon their results by these authors was incomplete. It appears possible that had Masson and his colleagues adopted their original plan for the intermittent administration of hydralazine by stomach tube this treatment might have proved as effective in controlling acute vascular disease as that which they selected, in spite of the varying blood pressure levels.

It was noticeable that treatment with intermittent hydralazine influenced equally the development of both arterial and arteriolar disease. This was thought to provide additional indirect evidence that both forms of vascular lesion were individual morphological components of the same response. It did not accord with the views of Bali and Goldblatt (1954) that the necrotizing arteritis and the arteriolitis of rats with renal hypertension were distinct.

The prevention of both lesions by treatment which led to fluctuating blood pressure suggested that the involved segments of arteries and arterioles in experimental rat hypertension were damaged by some mechanism dissociated from the absolute levels of systolic and diastolic blood pressure. For this reason it appeared desirable to search for the smallest amount of hydralazine which, given once daily, would prevent the evolution of acute vascular damage in animals with steroid hypertension. A further experiment was therefore performed. Albino male rats were subjected to the same steroid hypertensive regime, and were injected once daily with 1·0 mg., or with 0·2 mg. hydralazine (Fig. 11, Table IV). When the animals were killed at the end of 6 or 7 weeks treatment it was clear that the injections had not prevented acute vascular disease although the single 1·0 mg. injections had resulted in significant daily falls in systolic and in diastolic blood pressures.

It was therefore concluded that the dissociation between acute vascular disease and absolute blood pressure levels was not complete. Prevention of acute vascular damage by single daily injections of hydralazine was inevitably associated with some change in systolic and in diastolic blood pressures. Consideration

was given to the possibility that the prevention of acute vascular disease by hydralazine was a function of an overall reduction in the mean blood pressure, or that discontinuous hypertension, for a period of some hours each day, had not allowed the operation of a mechanism, the nature of which is at present unknown,

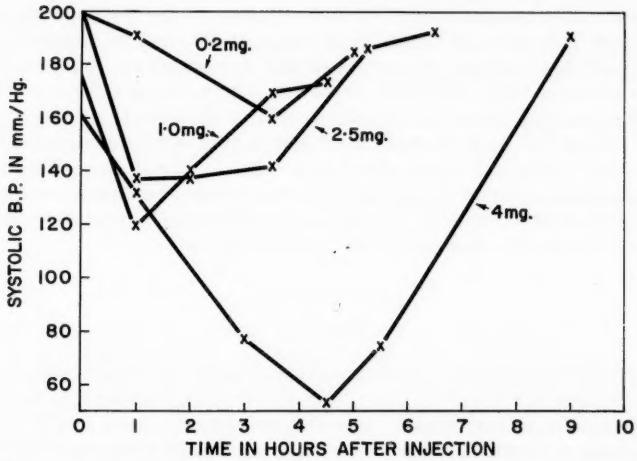

FIG. 11.—Response of the systolic blood pressure in an animal with steroid hypertension to varying doses of hydralazine injected intramuscularly.

which initiated acute vascular damage. Alternatively, and in accordance with the views of Goldbaltt (Bali and Goldblatt, 1954) the treatment adopted in the present experiment may have led to a constant influence upon some unknown function, possibly renal in nature, more sensitive to the action of hydralazine than was the blood pressure. In this way treatment may have controlled one of two

TABLE IV

Effect of Small Intermittent Dosage of Hydralazine

Group	Cortexone (deoxycortone)	Hydralazine daily	Duration of treatment	Mean weight change (g.)	Terminal mean weight (g.) of: R. kidney	Heart	Adrenals
7	Single 35 mg. subcutaneous implants	1 mg.	7 weeks	+34	2·32 (0·98%)	1·06 (0·45%)	0·066 (0·028%)
8		0·2 mg.	6 weeks	+60	2·92 (1·02%)	1·19 (0·41%)	0·076 (0·026%)

Incidence of Vascular Lesions

Group	Kidney	Adrenal	Heart	Lung	Mesentery	Pancreas	Lymph nodes	Liver	Spleen
7 (5)	5	0	0	0	5	5	0	1	2
8 (6)	4	0	0	1	3	3	1	2	3

factors necesary for the development of acute experimental hypertensive vascular disease, the other factor, raised blood pressure, being ineffective in isolation.

This experiment suggests that it should no longer be assumed that acute vascular disease in the rat with steroid hypertension, and perhaps with other forms of experimental hypertension, is the direct result of a critical rise in blood pressure alone. The relationship of this conclusion to the pathogenesis of hypertensive vascular disease in other animals and in man, and to the mode of action of other hypotensive drugs remains entirely speculative. Nevertheless, it is tempting to consider these results in reviewing the work of Leishman (1959) who observed that patients given a hypotensive drug (hexamethonium) by injection twice daily responded well to such treatment in spite of the difficulty of maintaining constantly lowered blood pressure levels. In the light of the present evidence it appears that this result may have been the consequence of an unexpectedly sustained and beneficial influence of such intermittent treatment on the evolution of hypertensive vascular disease and its complications.

SUMMARY

During the development of systemic hypertension in albino rats treated with salt and cortexone the small arteries of many organs became the site of a focal segmental necrotizing arteritis. These lesions were usually accompanied by a widespread focal arteriolitis often involving the afferent glomerular arterioles and characterised by the presence of subintimal material with the staining properties of fibrinoid. Both arterial and arteriolar lesions resembled morphologically those found in several other forms of experimental hypertension in the albino rat.

When rats were subjected to this regime of salt and cortexone and were simultaneously injected with increasingly large amounts of hydralazine once daily, neither form of vascular lesion developed during periods of observation which extended for up to 3 months.

The single daily injections of hydralazine caused severe repetitive daily fluctuations in both systolic and in diastolic blood pressures, leading to intermittent reduction in the mean systemic blood pressure. Each injection was followed by a rapid and profound fall in systolic and in diastolic pressure and was succeeded within 24 hr. by a slower rise to the pre-injection level.

These results show that the prevention of acute vascular disease in rats with steroid hypertension may take place in spite of repetitive abrupt daily rises in systolic and diastolic blood pressure. This evidence lends weight to the theory that a critical rise in blood pressure alone is not an adequate explanation for the appearance of acute arterial and acute arteriolar lesions in rats with experimental hypertension.

It is suggested, on the basis of these observations, that the necrotizing arteritis and the fibrinoid arteriolar disease of experimental rat hypertension are related phenomena, and that intermittent hydralazine prevents their development either by a discontinuous influence upon the mean blood pressure or by affecting a factor, at present unrecognised, which is more sensitive to the action of the drug than is the blood pressure.

In the absence of further evidence, caution must be exercised in translating the significance of these results to other animal species or to man.

This experiment was completed during the tenure of a Nuffield Travelling Fellowship at the Institute of Pathology, Western Reserve University, Cleveland, Ohio. To the Director of the Institute, Dr. A. R. Moritz, I wish to express my thanks for the many facilities made available.

The advice and criticism of Professor G. L. Montgomery are gratefully acknowledged.

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The photographs are the work of Mr. B. Brownlow, of the Institute of Pathology, Western Reserve University and Mr. D. N. Schad of Western Reserve University Medical School.

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IMMUNE HAEMOLYSIS IN RABBIT RADIATION-CHIMAERAS

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LOUTIT (1954) was the first to focus attention on the commonly transient nature of the recovery of lethally X-irradiated animals following treatment with homologous or heterologous bone marrow.

In rabbits, after initial recovery, there is a subsequent deterioration starting any time between 14 and 42 days after treatment, accompanied by wasting, diarrhoea, leucocytosis and often terminal anaemia and jaundice. The outcome of this " secondary disease " is variable ; in many it is lethal, but a few survive and gradually regain a semblance of normal appearance.

The striking difference between the results of isologous as opposed to homologous marrow treatment in mice has led to the conviction that these deaths are the consequence of an immunological reaction between host and graft.

Using the nuclear sex difference in the granulocytes as a biological cell label, Porter and Murray (1958) have shown that in rabbits late deaths are only rarely due to rejection of the marrow transplant by the host. Male chimaeras suffering from secondary disease continue to show female leucocytes in their blood indicating an intact and functioning marrow transplant. At necropsy the bone marrow is well repopulated or even hyperplastic, but there is widespread lymphoid atrophy, and in the spleen, which is in many cases enlarged, there is plasma cell infiltration.

The close resemblance both clinically and histologically between these animals and " runts " produced by injecting homologous adult spleen into foetal mice or chick embryos (Billingham and Brent, 1957 ; Simonsen, 1957) supports the suggestion of Trentin (1956) and others that death is due to reaction of grafted lymphoid cells against a host incapable of rejecting the foreign tissue.

Further evidence compatible with this concept has recently been accumulated. Results indicating lymphoid repopulation of the host from the injected marrow have been obtained (Ford, Hamerton, Barnes and Loutit, 1956 ; Barnes, Ford, Ilbery, Koller and Loutit, 1957) ; and Hobson, Porter and Whitby (1959) have demonstrated the production of antibodies by marrow cells transplanted from immunized donors into irradiated homologous rabbits. The serum γ-globulin of irradiated mice treated with rat bone marrow is now known to be of rat type (Weyzen and Vos, 1957 ; Grabar, Courcon, Merrill, Ilbery and Loutit, 1957), and it has been shown that radiation-chimaeras exhibit a long lasting tolerance to skin grafts of marrow donor origin. Such skin homografts or heterografts are usually in good condition at the time of death from secondary disease (Trentin, 1956 ; Zaalberg, Vos and van Bekkum, 1957).

If, as these facts imply, antibodies are produced in chimaeras by the grafted cells against host antigens, it would not be surprising if host red cells also suffered immune damage. That this happens in runt disease has been shown by Simonsen

(1957); that it might be a feature of secondary disease in rabbits is suggested by the occurrence of terminal anaemia and jaundice.

In this report evidence will be presented that in many rabbit radiation-chimaeras such an immune haemolysis of surviving host red cells does occur. Whether this is accompanied by obvious jaundice, however, depends upon the presence or absence of cirrhosis of the liver due to coccidial infection.

MATERIALS AND METHODS

Animals.—Young adult Chinchilla rabbits which were not inbred in the genetic sense were used throughout this study.

X-irradiation.—Total body X-irradiation was given as a horizontal beam to 2 animals at a time from a Westinghouse machine under the following conditions: 220 kV., 12·5 mA., 70 cm. target to skin distance, 1·0 mm. Cu and 1·0 mm. Al filters, H.V.L. 1·8 mm. Cu, dose rate to skin 33·4 r. per min., to centre of animal 21·1 r. per min. The dosage was divided; an initial dose of 600 r. at the centre of the animal was followed 24 hr. later by 500 r, and after another 24 hr. by a further 500 r., giving a total of 1600 r. (L.D. 100 30 days).

Adult bone marrow and foetal haemopoietic tissue.—Sterile suspensions of 20 day foetal rabbit liver and adult marrow were prepared as described earlier (Porter and Murray, 1958; Porter, 1959), and injected into the marginal ear vein of the recipient male rabbit 1–3 hr. following the last dose of irradiation and within 1 hr. of the death of the female donor.

Antibiotic therapy.—All rabbits were given tetracycline hydrochloride intramuscualrly 50 mg. per day for the first 2 weeks following irradiation.

Radiochrome tagging.—Red cell survival was measured with ^{51}Cr labelled cells. The radiochrome used was a sterile saline solution of radioactive sodium chromate, $Na_2{}^{51}CrO_4$, with a specific activity of 10 mc/mg. of chromium. Five microcuries of radioactivity and not more than 4 μg. of chromium metal were added to each ml. of blood treated. Four ml. of blood was drawn from the marginal ear vein immediately after irradiation and before marrow injection, heparinized, centrifuged and the red cells mixed with the correct volume of radiochrome solution. After gently agitating for 30 min. at room temperature the cells were washed 3 times in plasma-saline, and finally suspended in saline to a volume of approximately 4 ml.; 3 ml. of this suspension was injected. Samples of 0·3 ml. were taken at 1 hr. and 24 hr. and at appropriate times thereafter. Each sample was placed in a small tube containing 4 ml. of saline. The 1 hr. sample was counted with the subsequent samples, thus correcting for the physical decay of the chromium. Counting was carried out in a well type scintillation counter using a thallium activated sodium iodide crystal.

Anti-globulin reaction.—An adaptation of the anti-globulin reaction of Coombs, Mourant and Race (1945) was used. A suitable anti-rabbit globulin serum was made by immunizing guinea-pigs with alum precipitated rabbit globulin and following the methods used by Kellner and Hedal (1953). A quantitative direct Coomb's test for detecting the presence of coating antibody on the rabbit red cells was performed by making serial 4-fold saline dilutions of the antiglobulin serum, and then adding one drop of a 10 per cent red cell suspension to each dilution on an opalescent tile. The results were read after 5–7 min. and scored from $++++$ to $\pm$.

Bilirubin estimations were carried out using the method of Malloy and Evelyn (1937).

Haematology.—Reticulocyte and platelet counts and microhaematocrits were determined every 2 days. In all instances the establishment of a radiation-chimaera was confirmed by finding female type heterophils (amphophils) in an irradiated male rabbit (Porter, 1957).

Morbid anatomy.—All animals were necropsied at death or termination of the experiment.

RESULTS

Red Cell Survival Studies

A. *Normal non-irradiated rabbits*

Six normal rabbits were injected with a sample of their own red cells tagged with ^{51}Cr, and the curve of survival shown in Fig. 1 was obtained. The mean

apparent initial half time of these cells, *i.e.*, that time in which the ^{51}Cr activity decreased from 100–50 per cent, was 15 days which is in agreement with Waggener and Hunt's (1958) estimate, but is less than the 19 $\pm$ 5·9 days given by Marvin and Lucy (1957).

A further 3 rabbits were injected with a sample of ^{51}Cr labelled homologous blood. Donor and recipient were cross matched by the indirect globulin technique to avoid incompatibility. The curves of survival were essentially the same as those obtained after autologous blood and no real difference in apparent initial half time was detected.

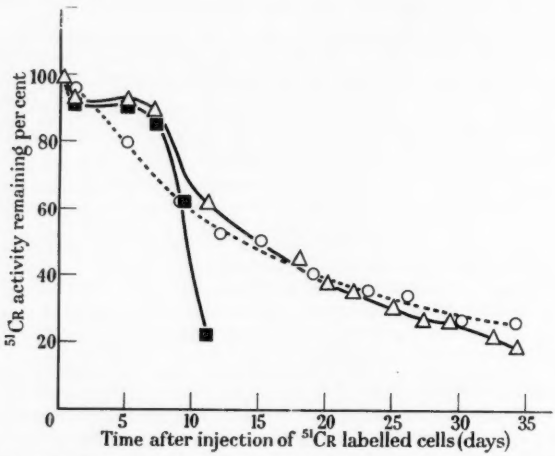


FIG. 1.—Survival of ^{51}Cr labelled autologous red cells in normal and lethally irradiated rabbits, and survival of labelled host erythrocytes in a radiation-chimaera produced by foetal liver.

○ - - - - ○ Normal non-irradiated rabbits (mean of 6).
■ ——— ■ Irradiated control.
△ ——— △ Irradiated rabbit treated with foetal tissue.

B. *X-irradiated but otherwise untreated rabbits*

The rate of ^{51}Cr loss following irradiation alone was determined in a group of 6 rabbits. In all animals there was an abrupt fall in the ^{51}Cr count in the first 24 hr. after irradiation (Fig. 1). Following this the count either remained unchanged for 4–6 days or else declined slowly. About the 7th day the ^{51}Cr count again began to fall rapidly and this process continued accompanied by ^{51}Cr in the faeces until the time of death. The mean apparent initial half time of these cells was 8 days which again is close to Waggener and Hunt's figure of 7·5 days.

In a further 3 rabbits the labelled cells injected were homologous. The curves of disappearance of ^{51}Cr from the circulation were the same as those obtained following transfusion of a sample of autologous blood and the initial half time was unaltered.

C. *Rabbit radiation-chimaeras produced by foetal tissue*

It has recently been shown (Porter, 1959) that lethally irradiated rabbits treated with 20 day foetal liver are protected from early death by rapid repopu-

lation of their depleted haemopoietic tissues by the injected cells. These animals also avoid secondary disease probably because the immunologically immature transplanted foetal cells become, as they mature, tolerant of the antigens of their host.

Red cell survival in 6 such chimaeras was studied after reinjection of ^{51}Cr labelled cells (Fig. 1). All animals in the group showed the same initial abrupt fall in ^{51}Cr count followed by a period during which little loss occurred, as was encountered previously in Group B. About the 7th day the ^{51}Cr count again began to fall rapidly, but this slowed by about the 12th day and from then onwards the rate of loss of ^{51}Cr was only a little faster than that for normal rabbits.

D. *Rabbit radiation-chimaeras (produced by adult tissue) in whom secondary disease developed*

Nine such rabbits were studied. In 6 a sample of their own blood was labelled and reinjected; in the remaining 3 homologous blood from the marrow donor was tagged and given.

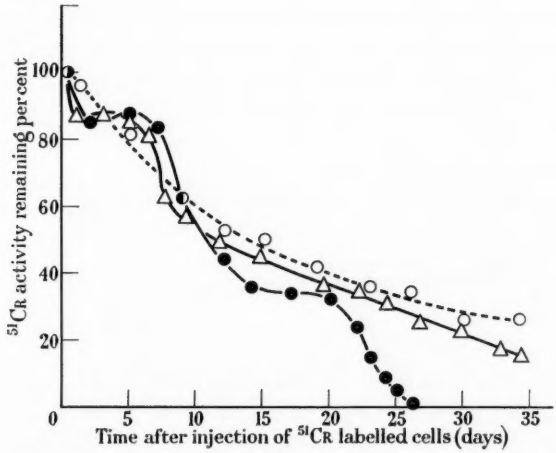


FIG. 2.—Survival curves for ^{51}Cr labelled donor and host red cells in a radiation-chimaera suffering from secondary disease. The curve for normal rabbits is included for comparison.

○ - - - - ○ Normal non-irradiated rabbits (mean of 6).
△ ——— △ Radiation-chimaera in whom secondary disease developed. Donor cells labelled.
● ——— ● Radiation-chimaera with secondary disease. Host cells labelled. Note the haemolysis commencing at the 20th day.

i. *Donor cells labelled.*—All animals in the group showed curves of disappearance of ^{51}Cr from the circulation (Fig. 2) identical with those found in the foetal tissue chimaeras given labelled host red cells (Group C).

ii. *Host cells labelled.*—Although these 6 animals showed the usual initial abrupt fall in ^{51}Cr count followed by a plateau and then by a second fall, they

were distinguished by the occurrence of a third sudden fall commencing at any time between the 20th and 30th day after injection of the labelled cells (Fig. 2). Within a period of 5–7 days the ^{51}Cr activity disappeared completely.

Reticulocyte counts

Following irradiation reticulocytes rapidly vanished from the peripheral blood. In the irradiated controls (Group B) they never reappeared, but in all the radiation-chimaeras the normal level was exceeded in an upward surge on the 5th–8th day after treatment with haemopoietic tissue. Later they declined and only again appeared in large numbers in those rabbits which developed secondary disease (Fig. 3).

Haematocrit levels

After irradiation there was a drop in packed cell volume during the first 4 days. With the onset of thrombocytopenia between the 7th–10th days there was in the irradiated controls (Group B) a rapid fall in haematocrit, continuing until

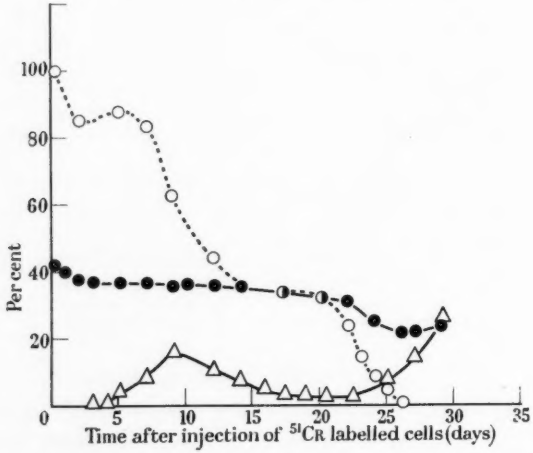


FIG. 3.—Reticulocyte counts and haematocrit values in a rabbit radiation-chimaera dying from secondary disease. The survival curve for ^{51}Cr labelled host red cells from the same animal is included for comparison.

△ ——— △ Reticulocytes (per cent).
● ——— ● Haematocrit (per cent).
○ - - - - ○ ^{51}Cr activity remaining (per cent).

death. In the foetal-tissue treated rabbits (Group C) this precipitous drop did not occur and the haematocrit after sinking slowly until about the 12th day, then began very slowly to rise. Radiation-chimaeras treated with adult marrow at first followed a similar course to those treated with foetal tissue, but with the onset of secondary disease a rapid profound fall in the packed cell volume occurred (Fig. 3).

Platelet counts

Severe thrombocytopenia occurred in the irradiated controls between the 7th and 10th day accompanied by a generalized haemorrhagic tendency. In the animals treated with haemopoietic tissues an initial fall in platelets was checked by the 6th day and from then onwards the count rapidly returned to normal levels.

Blood films

Animals suffering from secondary disease (Group D) showed as the anaemia progressed increased numbers of polychromatic macrocytes, microspherocytes, nucleated red cells and irregularly distorted erythrocytes.

Quantitative direct antiglobulin test

As can be seen from the table, samples of washed red cells obtained from radiation-chimaeras immediately the plasma first became tinged with yellow or the animal developed signs of secondary disease, gave a positive direct Coomb's test. The strength of agglutination varied from animal to animal but was definite in all cases.

Bilirubin estimations and incidence of jaundice and biliary cirrhosis

During this investigation it became apparent that whilst all the radiation-chimaeras suffering from secondary disease showed evidence of haemolysis with increased serum bilirubin, only 5 (Table) became jaundiced. Further, total

TABLE.—*Serum Bilirubin Levels, and Dilutions of Antiglobulin Serum giving Maximum Agglutination of Red Cells, in Rabbit Radiation-chimaeras Suffering from Secondary Disease*

Radiation chimaera	Jaundice	Biliary cirrhosis	Serum bilirubin (mg. per cent) maximum reading			Quantitative direct Coomb's test	
			Total	Direct	Indirect		
1	+	+	1·1	0·7	0·4	1/4	+
2	+	+	3·2	1·6	1·6	1/64	+
3	—	—	0·9	0·2	0·7	1/4	++
4	+	+	3·0	1·6	1·4	1/64	++
5	—	—	0·7	0·1	0·6	1/16	++
6	+	+	2·1	1·2	0·9	1/16	+++
7	—	—	0·7	0·2	0·5	1/64	+
8	—	—	1·0	0·2	0·8	1/4	+
9	+	+	2·4	1·4	1·0	1/256	++
Normal rabbits	No	No	0·1	0·1	0·0	0	—

bilirubin levels were not only considerably higher in animals with clinical jaundice, but the direct component exceeded the indirect. Jaundice was not encountered in any of the other rabbits used in these experiments. At necropsy each of the jaundiced chimaeras showed a severe biliary cirrhosis with marked periportal and perilobular fibrosis and numerous newly formed bile ducts (Fig. 4). Stasis of bile was often present. In 2 cases the epithelial cells of the proliferating bile ducts contained merozoites of coccidiosis; in 1 case a few oocysts were found. No

parasites were seen in the other 2 cases. Examination of the gall bladder bile revealed oocysts of *Eimeria stiedae* in 4 of the 5 jaundiced animals. Isolated coccidiosis lesions were found in the livers of 9 of the remaining 24 animals used in these experiments. Biliary cirrhosis, however, was confined to the group of rabbits with secondary disease.

Haemosiderosis of the reticulo-endothelial system

Three rabbits were irradiated and then 1 animal was killed on the 2nd, 4th and 6th days after their last exposure to the X-rays. The spleen, liver, bone marrow and lymph nodes were stained for iron by the Prussian blue method. Haemosiderin was present in considerable quantities in the spleen, bone marrow and, to a lesser extent, the liver in the animal killed on the 2nd day. By the 4th day the iron deposits were much heavier ; and on the 6th day small deposits in the lymph nodes were apparent. Chimaeras with secondary disease showed very heavy reticuloendothelial haemosiderosis, particularly in the spleen (Fig. 5) where erythrophagocytosis was also present. Iron deposition was much less in the chimaeras which did not develop secondary disease.

DISCUSSION

In all the irradiated rabbits there was a rapid loss of ^{51}Cr in the first few days. This phenomenon has been noted previously in irradiated dogs by Stohlman, Brecher, Schneiderman and Cronkite (1957) and is poorly understood. That it represents destruction of red cells and not just dilution by an initial outpouring of erythrocytes into the circulation, is shown by the early accumulation of haemosiderin in the spleen and elsewhere in the reticuloendothelial system. A direct haemolytic effect of irradiation on the red cells is unlikely to be important in this early phase for a similar chromium loss occurs when normal autologous cells are transfused immediately after irradiation (Stohlman *et al.*, 1957).

The slow removal of ^{51}Cr observed from the 4th to the 6th days is compatible with slow loss of red cells by ageing in an animal in which red cell production has ceased.

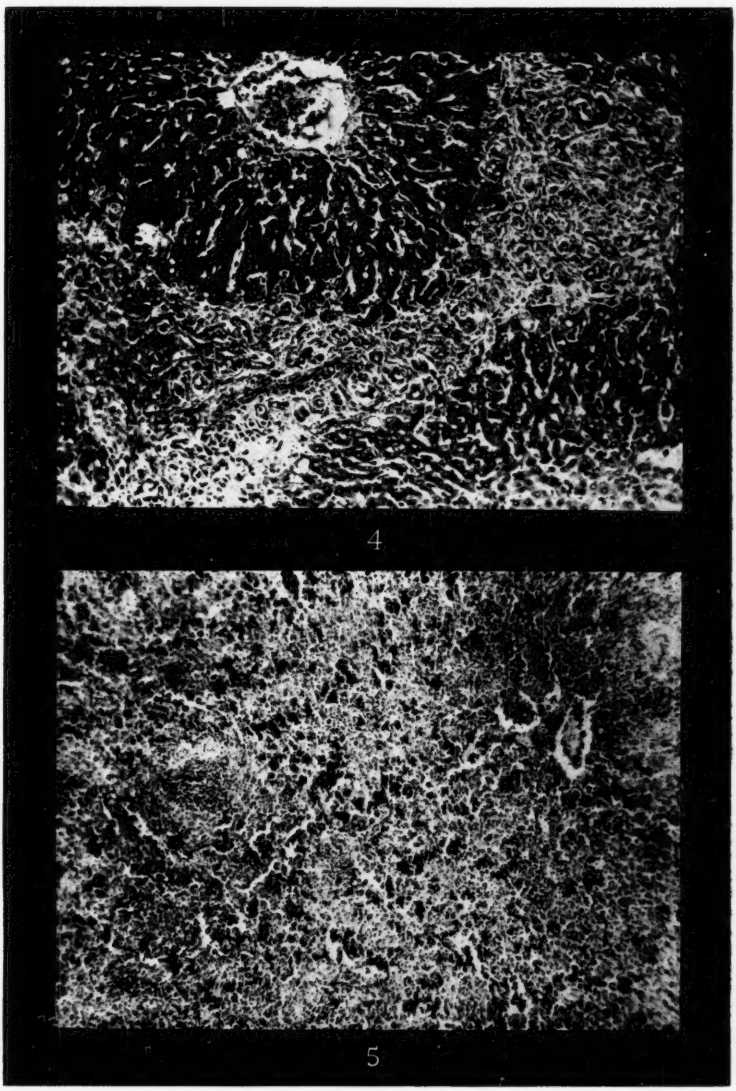
At the 7th day there followed in the irradated control rabbits, a second rapid fall in the ^{51}Cr count due mainly to extravasation of red cells into the tissue and lymph spaces (Kahn and Furth, 1952) and haemorrhage into the gut. These effects were associated with capillary damage and thrombocytopenia and culminated in death of the animal.

In rabbits treated with haemopoietic tissue this second rapid loss of ^{51}Cr activity was neither so precipitous nor so prolonged as in the irradiated controls and it coincided with a rapid rise in the reticulocyte count. In these cases an important factor was probably dilution of the ^{51}Cr labelled cells by an outpouring of newly formed untagged cells from the marrow transplant. By the 12th day the ^{51}Cr loss

EXPLANATION OF PLATE

FIG. 4.—Liver of jaundiced rabbit dying from secondary disease. Note bile duct proliferation and fibrosis in portal tracts. Examination of bile showed *E. stiedae*. Haematoxylin and eosin. × 60.

FIG. 5.—Haemosiderin laden spleen of a rabbit which died from secondary disease. Prussian blue. × 60.



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had slowed and from then onwards the slopes of the survival curves in those animals treated with foetal tissue and in those transfused with donor labelled cells were similar to those obtained for normal rabbits and reflect normal senescence.

The advent of secondary disease in certain chimaeras was associated with a third abrupt fall of the ^{51}Cr count continuing this time to zero. The associated reticulocytosis, spherocytosis, drop in packed cell volume, increased indirect serum bilirubin concentration and splenic haemosiderosis with erythrophagocytosis, all favour this loss of chromium being due to destruction of red cells. Moreover, this haemolysis has been shown to be selective in that only host erythrocytes were destroyed. Transfused donor cells were unaffected. Finally the positive direct antiglobulin reaction obtained in each of the radiation-chimaeras with secondary disease demonstrates the presence of antibody globulin attached to the surface of some of the red cells. That the indirect Coomb's test may also sometimes be positive is indicated by the work of Lee, Dealy, Dammin and Brooke (1958).

This evidence suggests that an important feature of secondary disease in the rabbit is an immune haemolysis of surviving host red cells. It seems reasonable to speculate that the following sequence of events takes place. The bone marrow used for treatment of the lethally irradiated animal includes some primitive cells capable of recolonising the severely damaged lymphoid system of the host. Because irradiation has destroyed the host's ability to reject foreign tissue these cells after successfully implanting are free to respond unchecked to the antigenic stimulus presented them by the host. They multiply, establish a new immune system and begin to elaborate antibodies against host antigens, including red cell antigens.

The situation is therefore strikingly comparable to that obtaining when a late chick embryo has been injected intravenously with homologous adult spleen cells (Simonsen, 1957). Here too the host is immunologically defenceless, not because of irradiation damage but simply by virtue of the immaturity of the immune system. The injected donor cells proliferate, repopulate the lymphoid system of the host and proceed to attack the host cells. After hatching the chick develops a severe immune-haemolytic anaemia and a positive direct Coomb's test.

Anaemia due to haemolysis has also been noted in parabiosis intoxication (Mayeda, 1921) and in the " wasting disease " which occurs when parental haemopoietic cells are injected into F_1 hybrid mice (Trentin, 1958). Again the host is unable to destroy the attacking foreign lymphoid cells because of immunological paralysis in the first instance and genetic considerations in the second.

Jaundice, the feature which originally attracted attention to the possibility of haemolysis occurring in rabbits dying from secondary disease, has been shown to occur only if there is in addition parasitic liver damage. Coccidiosis is an extremely common disease in the rabbit, even in apparently healthy stock. Most adults, however, become immune and any lesions are usually confined to the occasional encapsulated nodule containing degenerated oocysts. Radiation chimaeras with their impaired capacity for producing antibodies, and particularly those with secondary disease, are obvious candidates for a coccidial recrudescence or reinfection. As we have seen one of the manifestations of secondary disease is haemolysis of host red cells causing a sudden rise in serum bilirubin. Although not icteric in normal rabbits this increase in circulating bile pigment will precipitate jaundice in any animal with coccidial liver damage.

SUMMARY

In rabbit radiation-chimaeras which develop secondary disease there is a rapid destruction of ^{51}Cr labelled host erythrocytes.

This haemolysis is only seen in association with secondary disease and does not occur in chimaeras produced by treatment with foetal tissue.

The destruction of red cells is selective, labelled donor cells remaining unharmed.

The accompanying positive direct Coomb's test suggests that the haemolysis is an immune one.

These facts strongly support the hypothesis that this haemolysis, in common with the whole syndrome known as secondary disease, is due to an immune reaction by the graft against the host.

Jaundice will appear in the ailing chimaera only if there is also cirrhosis of the liver due to coccidiosis.

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IMMUNITY IN *MYCOBACTERIUM LEPRAEMURIUM* INFECTIONS

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IN a previous investigation on infections of the mouse cornea with *Mycobacterium lepraemurium*, it was found that the organisms multiplied rapidly in the cornea for about 6 weeks, but thereafter multiplication was at a much slower rate (Robson and Smith, 1959). It appeared possible that the slowing down was due to the development of immunity and the present investigation was performed to test this; at the same time, the possible value of *Mycobacterium tuberculosis* (B.C.G.) immunization was investigated.

MATERIALS AND METHODS

White mice weighing about 20 g. at the beginning of the experiment were used and were inoculated intracorneally as described by Rees and Robson (1950). The suspension of *Myco. lepraemurium* used for inoculation was prepared as described by Naguib and Robson (1956) from mice that had previously been infected by the intravenous route. The lesions which developed in the cornea were investigated at various stages after inoculation as follows:

Quantitative estimation of the total number of mycobacteria in the cornea, as described by Robson and Smith (1959). In the present investigation such counts were always done on 2 corneas so that each point on a curve represents the average of 2 corneal estimations: size of the macroscopic lesion as followed quantitatively by Rees and Robson (1950) and Naguib (1956); staining of the whole cornea by a modified Ziehl Neelsen technique as described by Robson and Sullivan (1957).

For the main control group 22 mice were taken and injected intracorneally with about 10 million organisms and the progress of this infection was followed by counting the organisms in the corneas of 2 mice at weekly intervals. At the same time, 2 groups of 36 mice were immunized intradermally, the first group with 300 million *Myco. lepraemurium* organisms (total count) and the other group with 100,000 viable units of B.C.G. After 12 days, when the organisms were still multiplying rapidly in the main control group (Fig. 2), half of each vaccinated group, together with 18 normal mice included as controls, were challenged intracorneally with about 10 million *Myco. lepraemurium* organisms. The multiplication of the organisms of each of these 3 groups was also followed by counting 2 corneas from each group at weekly intervals. After 74 days from the start of the experiment, when the rate of multiplication of the organisms in the main control group had slowed down considerably (Fig. 2), the remainder of the 2 groups of unchallenged vaccinated mice, together with 18 normal animals included as controls, were challenged intracorneally with about 10 million *Myco. lepraemurium* organisms. The multiplication of the organisms in each of these 3 groups was similarly followed by counting 2 corneas from each group at weekly intervals.

RESULTS

The development of macroscopic corneal lesions in the various groups is shown in Fig. 1 and the multiplication of organisms in Fig. 2. It will be seen that vaccination, either with *Myco. lepraemurium* or with B.C.G. did not significantly slow down the development of macroscopic lesions, this being true whether

the animals were challenged 12 or 74 days after vaccination. Thus the vaccination had no effect on the development of macroscopic lesions in the cornea. A marked difference was, however, seen when the multiplication of organisms in the cornea was taken as a criterion. In the animals challenged 12 days after vaccination, at a stage when no immunity appeared to have developed as judged by the result in the main control group, the multiplication of organisms in both vaccinated groups was practically the same as in the appropriate control. On the other hand, in the animals challenged 74 days after vaccination when multi-

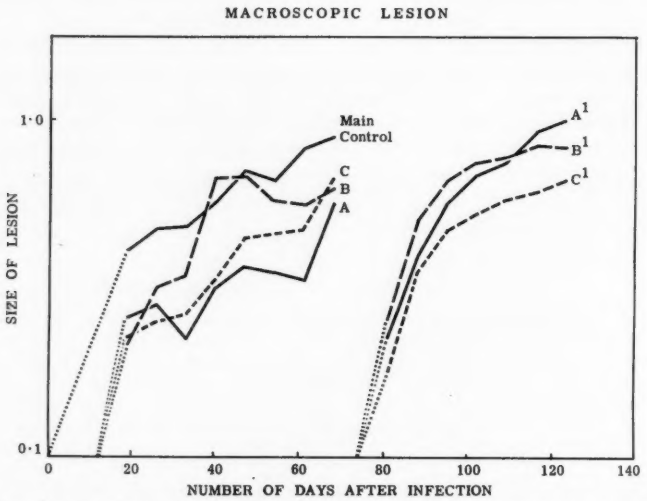

FIG. 1.—Showing the effect of vaccination on the development of macroscopic lesions in the cornea (logarithmic scale). The main control group illustrates the development of lesions in unvaccinated animals. Vaccinated mice were challenged at 12 or 74 days after vaccination and the development of lesions in these groups and their appropriate controls is shown.

A Control for mice challenged 12 days after vaccination.
B Mice vaccinated with *Myco. lepraemurium* and challenged after 12 days.
C Mice vaccinated with B.C.G. and challenged after 12 days.
A^1 Control for mice challenged at 74 days after vaccination.
B^1 Mice vaccinated with *Myco. lepraemurium* and challenged after 74 days.
C^1 Mice vaccinated with B.C.G. and challenged after 74 days.

plication had considerably slowed in the main control group, the vaccination had a striking effect, so that the rate of multiplication in the corneas of both vaccinated groups was considerably less than in the appropriate control. Indeed, at the end of the experiment, the number of organisms in the corneas of both groups of vaccinated mice was not appreciably different from that actually inoculated whereas in the controls they had multiplied more than 10 times.

In the animals challenged 74 days after vaccination there was thus a marked discrepancy between the effect of vaccination on the development of corneal lesions and on the multiplication of organisms. This is illustrated in Fig. 3, in which it can be seen that for equivalent lesions, many more organisms were present in the control than in the vaccinated groups.

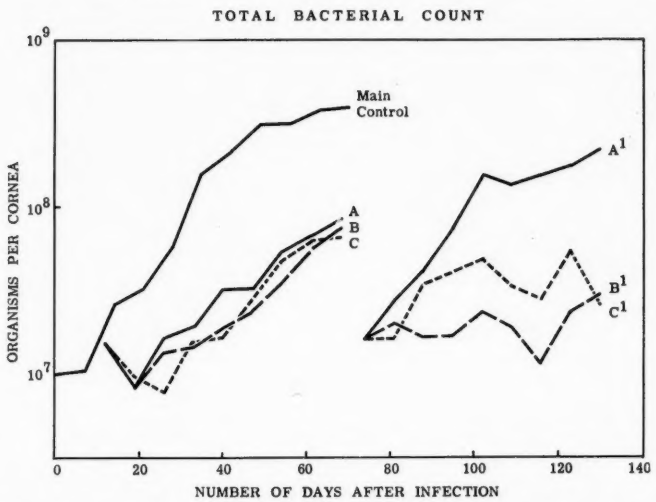

FIG. 2.—Showing the effect of vaccination on the multiplication of the *Myco. lepraemurium* in the cornea (logarithmic scale). The main control group illustrates the multiplication of the organisms in unvaccinated animals. Vaccinated mice were challenged at 12 or 74 days after vaccination and the multiplication of the organisms in these groups and their appropriate controls is shown.

A Control for mice challenged 12 days after vaccination.
B Mice vaccinated with *Myco. lepraemurium* and challenged after 12 days.
C Mice vaccinated with B.C.G. and challenged after 12 days.
A^1 Control for mice challenged 74 days after vaccination.
B^1 Mice vaccinated with *Myco. lepraemurium* and challenged after 74 days.
C^1 Mice vaccinated with B.C.G. and challenged after 74 days.

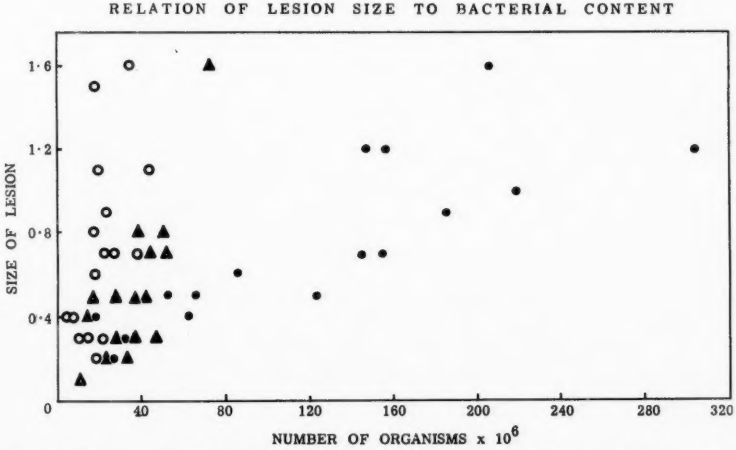

FIG. 3.—Showing the number of *Myco. lepraemurium* in relation to the lesion size in :

● Unvaccinated mice.
○ Mice vaccinated with *Myco. lepraemurium* and challenged 74 days later.
▲ Mice vaccinated with B.C.G. and challenged 74 days later.
Each point represents the bacterial count and the lesion size for a single cornea.
The mice were killed at various periods after inoculation.

DISCUSSION

These experiments show that when mice were vaccinated intradermally either with *Myco. lepraemurium* or with B.C.G. and then challenged 12 days later intracorneally with *Myco. lepraemurium* there was no evidence of any immunity, as judged both by the development of macroscopic lesions and by the multiplication of the mycobacteria in the cornea. These results are, therefore, in agreement with the finding that at that stage mycobacteria were actively multiplying at a primary site of inoculation (the cornea), though it is possible that a challenge with a smaller inoculum might have demonstrated some degree of immunity. When the challenge with the large inoculum was made 74 days after vaccination, then immunity could be demonstrated by the failure of the mycobacteria at the challenged site to multiply to any appreciable extent. This result therefore supports the view that the slowing down of bacterial multiplication at the primary site is due to immunity. Immunity against infection with *Myco. lepraemurium* has been previously described by Hanks and Fernandez (1956) who investigated leproma formation in rats and found that both *Myco. lepraemurium* and B.C.G., and better still a combination of the 2 organisms, decreased the incidence and development of lepromata.

What was rather puzzling in our own experiments was that in the corneas challenged 74 days after vaccination there was marked inhibition of bacterial multiplication, as compared with unvaccinated controls, yet the development of macroscopic lesions was not significantly affected. That such a lesion is not merely the result of a progressively foreign body reaction to the inoculum, independent of any biological effect of the organisms, was shown by Naguib, Rees and Robson (1956) in that inoculation of heat killed mycobacteria into the cornea evoked only minimal lesions which did not increase in size over a period of many weeks.

The most likely explanation for these results seems to be that the large live inoculum (about 10^7 organisms) introduced into the cornea exerted and continued to exert a chemotactic influence much more marked than that produced by the heat killed organisms, and that the cellular reaction led to the production of the macroscopic lesion. In the animals not previously immunized the bacilli continued to multiply, essentially within the phagocytes, while in the previously immunized mice such multiplication was much less, so that the organisms, though contained within the cells which essentially constitute a macroscopic lesion similar in size to that seen in non-immunized animals, are fewer in number than in the non-immunized animals. It may well be that in the previously immunized animals it was the phagocytes which limited the multiplication of the bacilli, a view in keeping with some previous experimental results though not with others (Raffel, 1955). It is interesting that Conge, Collin, Levy and Dubos (1959) found that B.C.G. vaccination did not decrease the number of pulmonary lesions elicited in mice by the intravenous injection of *Myco. tuberculosis*, though it decreased considerably the number of bacilli detectable by microscopy in each lesion; unfortunately these investigators make no mention of the size of the lesion found in these animals. It is also of interest that somewhat progressive lesions can be produced by H37Ra, though this avirulent organism does not multiply (Oatway and Steenken, 1936).

Another, or additional, possible explanation for our results requires discussion, namely that immunity, by restricting the spread of the bacilli from the main corneal lesion, kept down the total number of organisms present in the immune animals, whereas in non-immune mice such spread, though not contributing to the visible macroscopic lesion, would greatly add to the total number of bacilli recoverable from any particular cornea. We have stained the whole cornea of a small number of immunized and non-immunized animals with the modified Ziehl Neelsen technique in order to examine the spread of organisms and the results so obtained support the view that spread of organisms outside the macroscopic lesion is more extensive in the non-immunized mice, a feature of immunity described by Koch (1891) for tuberculosis. Further experimental evidence on this aspect of the work is, however, required.

SUMMARY

Mice were vaccinated intradermally with either *Myco. lepraemurium* or B.C.G. and challenged intracorneally with *Myco. lepraemurium* at either 12 or 74 days after vaccination. Vaccination had no effect on the development of lesions or the multiplications of the organisms when the mice were challenged 12 days after vaccination. However, when the mice were challenged 74 days after vaccination, the multiplication of the organisms was much less in both the B.C.G. and *Myco. lepraemurium* vaccinated groups than in the controls, but no difference was found between either vaccinated group and the controls as regards the lesion sizes.

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THE EFFECT OF FREUND'S ADJUVANTS ON LYMPHATICS IN THE MOUSE'S EAR

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FREUND'S adjuvants have been used in many studies for the purpose of enhancing antibody production. The adjuvant mixture, in which the antigen is incorporated in a water-in-oil emulsion containing killed *Mycobacterium tuberculosis*, has also been described as having a stimulant effect on reticulo-endothelial tissue. A preparation containing tubercle bacillary wax was shown to produce widespread proliferation of plasma-cell elements in guinea-pigs (White, Coons and Connolly, 1955) and recently reticulo-endothelial changes with the adjuvant have been described in guinea-pigs, mice and Syrian hamsters (Laufer, Tal and Behar, 1959). It seemed possible that a further effect of the adjuvants might be to induce lymphatic proliferation, and the present study was undertaken to investigate this possibility.

MATERIAL AND METHODS

Thirty adult male albino mice were randomly allocated to 3 treatment groups A, B and C, which were given injections of saline, turpentine and Freund's adjuvant emulsion respectively. The mice were anaesthetized with ether and 0·005 ml. of the requisite fluid was injected subcutaneously in the centre of the right ear with an Agla micrometer syringe through a gauge 30 platinum-iridium needle. The left ear served as an uninjected control in each animal.

The adjuvant emulsion consisted of 2 volumes of saline, 1 volume of Arlacel A, 2 volumes of Bayol F and 1 mg. killed tubercle bacilli per ml. of emulsion. (Freund, Thomson, Hough, Sommer and Pisani, 1948.)

After injection the animals were left for 27–29 days and their ears were then examined for evidence of lymphatic proliferation. They were anaesthetized with pentobarbitone (6mg./ml., 0·4 ml. per 25 gm. body wt.), laid on a bed of plasticine, and their ears gently spread on wax moulds. The ears were observed with reflected light under a Zeiss binocular dissecting microscope at a magnification of × 10. To show up the lymphatics, Indian ink was injected through an Agla microinjection syringe with a quartz microneedle. The needle was introduced repeatedly into the skin and a very small amount of Indian ink injected each time, until it entered a lymphatic, when 0·005–0·02 ml. of Indian ink was injected.

For photography, a coverslip was mounted over the ear, and liquid paraffin was run into the space between the ear and the coverslip. An orange filter was used to increase contrast between blood vessels and lymphatics and exposures of 6 sec. were made on Pan F or FPa film with a 35-mm. camera. Both ears of each of the 30 mice were injected with Indian ink, observed, and photographed.

RESULTS

On examination of the surface of the ears with the naked eye and under magnification of 5× the following were observed :

Group A (saline).—In the 10 mice in this group no trace of the original injection site was seen. Six animals showed slight irregularity of the edge of their ears

or small scattered surface lesions on one or both ears, presumably due to scratching or fighting.

Group B (*turpentine*).—Lesions were noted in the right ears of all 10 mice in this group. These varied from mild oedema and congestion of vessels in the centre of the ear in 3 mice, to actual perforation or notching of the ear in 7 mice. The left ears were normal except for a few tiny surface lesions in 2 animals.

Group C (*Freund's adjuvants*).—In this group of 10 mice there was no obvious damage to the right ears. In most animals the site of injection could be seen on careful inspection as a slightly thickened area in the centre of the ear. There was no distortion of the vascular pattern and in only 1 mouse was there any congestion or increase of small blood vessels. Five mice showed slight irregularity of the edges of the ears. The left ears were normal except for irregularities of the edge of the ear in 2 mice.

On injection with Indian ink the following were observed :

Group A (*saline*).—The normal lymphatic pattern was seen in both test and control ears (Fig. 1), except in the case of 1 mouse in which the right ear showed patchy proliferation of lymphatics associated with some damage to the skin.

Group B (*turpentine*).—There was diffuse proliferation of lymphatics in the test ears as compared with the control ears (Fig. 2). This varied considerably from animal to animal, some showing a fairly dense meshwork of lymphatics and some showing only moderate proliferation.

Group C (*Freund's adjuvants*).—In 8 out of the 9 mice there was, in the test ear, a circumscribed area of very marked proliferation of lymphatics at the site of the original injection, showing up as a dense meshwork of vessels (Fig. 3). In the remaining mouse, only moderate proliferation at the site of the original injection was seen.

DISCUSSION

The effect of Freund's adjuvants on lymphatic proliferation has been compared with the effect of turpentine, chiefly because turpentine was one of the substances used by Pullinger and Florey (1937) in their classical studies on lymphatic proliferation. It is interesting to note that the proliferation of lymphatics in the turpentine-treated animals accompanied some degree of macroscopic damage of the ear, *i.e.* the proliferation was associated with either the healing of an ulcer or a perforation, or with an active inflammatory process, as evidenced by congestion and oedema. The proliferation of lymphatics in the mice treated with Freund's adjuvants, however, occurred without exception in the absence of marked macroscopic damage. The ears of these mice appeared to be normal on naked eye inspection, the thickening at the site of the original injection being obvious only on examination under the dissecting microscope.

The question of whether the lymphatic proliferation induced by Freund's adjuvant assists in the enhancement of antibody production is beyond the scope of this paper. The effect of adjuvants in potentiating antibody production is ascribed partly to retardation of the absorption and elimination of the antigen (Freund, 1947), partly to a general proliferation of plasma cell elements (White, Coons and Connolly, 1955) and partly to the production of a local granuloma containing antibody-producing cells. It is possible that a further factor may be proliferation of local lymphatics and thus preferential absorption of the antigen

into the lymphatic system rather than the general circulation. Gladstone and Abrahams (1958), discussing the possible significance of the local response in explaining the adjuvant effect, say " . . . since much of the antibody is formed in the regional lymph glands draining the site of injection, it might be argued that dispersal to lymph glands rather than local retension, was desirable." It is suggested that it might be worthwhile to investigate the effect of introducing adjuvants at a particular site a week or two before an immunizing injection, with the idea of preparing a " bed " of lymphatics in the area to receive the antigen. On the basis of the present experiments, however, one can go no further than to say that lymphatic proliferation occurs with Freund's adjuvants in the mouse's ear. It would be of interest to ascertain whether this proliferation is produced by one of the constituents of Freund's mixture or whether it is a function of their acting in combination.

SUMMARY

Lymphatic proliferation was studied in the ears of mice which had been injected with saline, turpentine or Freund's adjuvants a month previously. In mice injected with Freund's adjuvants, the ears showed little macroscopic damage but marked local proliferation of lymphatics at the site of injection. Mice injected with turpentine showed fairly extensive damage and varying degrees of lymphatic proliferation. Mice injected with saline showed no change from the normal pattern of lymphatics.

I wish to thank Professor T. Gillman for his encouragement. I am indebted to the Council of Scientific and Industrial Research for financial support. My thanks are also due to Mr. R. Govender and Mr. A. S. Rangiah for technical assistance and to Mr. R. Kirk for photographic assistance.

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EXPLANATION OF PLATE

Photographs of mouse ears after injection with Indian ink, taken with reflected light at a magnification of 5 × using an orange filter.

(*a*) Left ears. (*b*) Right ears.

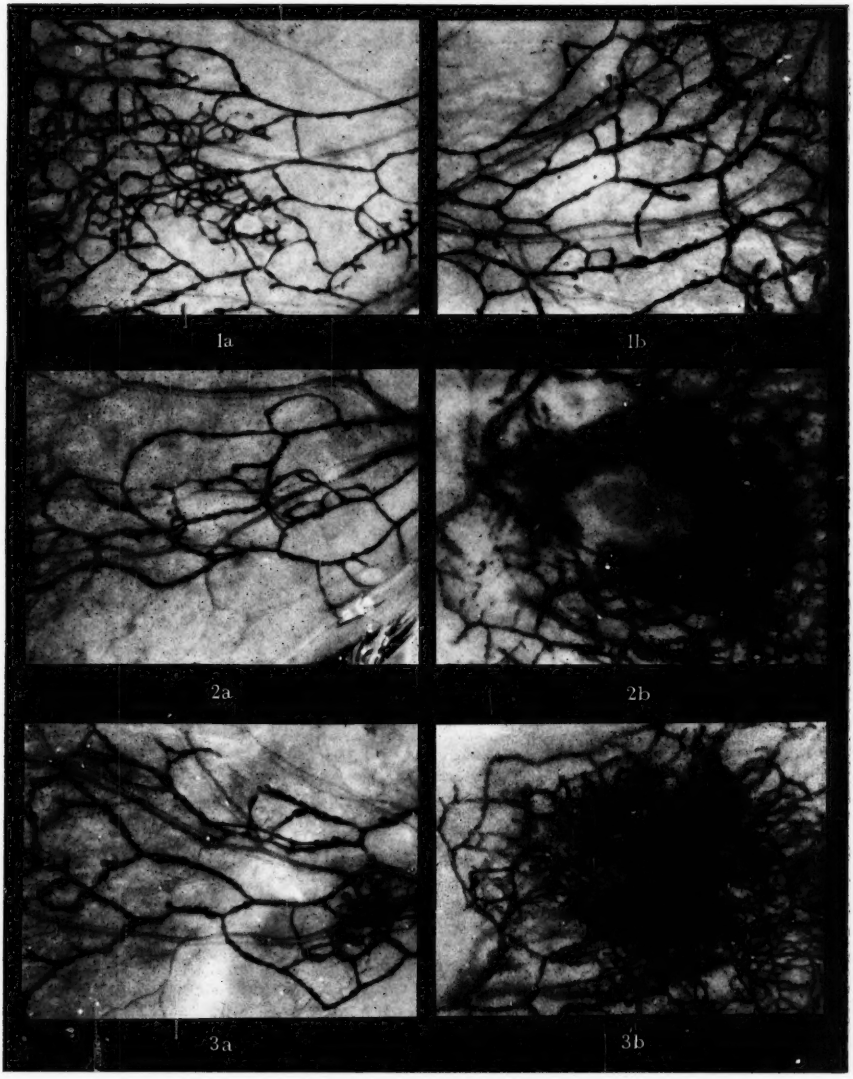
The right ears had been injected 29 days previously with saline (No. 1), turpentine (No. 2) or Freund's adjuvants (No. 3). The left ears were controls.

No. 2 (*b*) shows a perforation in the centre of the ear.

FIG. 1.—*a*—Control. *b*—Injected 29 days previously with saline.

FIG. 2.—*a*—Control. *b*—Injected 29 days previously with turpentine.

FIG. 3.—*a*—Control. *b*—Injected 29 days previously with Freund's adjuvants.


1a
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3b

ANTIBODY PRODUCTION BY SINGLE CELLS

IV: Further Studies on Multiply Immunized Animals

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Earlier work from this laboratory (Nossal and Lederberg, 1958; Nossal, 1958) suggested that no cell will produce more than one type of antibody at one time. In more recent studies of antibody production by single cells (Nossal, 1959*a*; 1959*b*) more satisfactory techniques for preparing single cell microdroplets were described. A better method for testing microdroplets for the presence of 2 or 3 unrelated antibodies is now reported. Using this method, we have tested 1399 lymph node cells from animals immunized in various ways with 2 or 3 unrelated antigens. The finding of phenotypic restriction of antibody-producing capacity of lymph node cells has been confirmed.

MATERIALS AND METHODS

Animals.—Adult male Wistar albino rats were used. They were fed on a diet of mouse pellets and tap water *ad libitum*.

Bacteria.—Four serologically unrelated monophasic strains of Salmonella were used. They were *Salmonella adelaide*, (H_1^{g}), obtained from the Department of Public Health, University of Melbourne, through the courtesy of Mr. G. Cooper; 2 transductional derivatives of *Salmonella paratyphi B*, Sw 685, (H_1^{g}) and Sw 997 (H_1^{f}); and 1 transductional derivative of *Salmonella typhi* Sw 940 (H_1^{b}). These 3 strains were kindly supplied by Professor Joshua Lederberg from his collection at Madison, Wisconsin. These bacterial strains and antigens derived from them will be referred to as AD, 685, 997 and 940 respectively. Bacteria were maintained at maximal motility by frequent passage through a semi-solid nutrient gelatin agar medium (Lederberg, 1956) and fresh 3–4 hr. broth cultures were used for motility inhibition tests.

Antigens.—Semi-purified flagella were prepared from each strain as previously described (Nossal, 1959*c*). About 0·5 ml. of flagellar material was dissolved in 10 ml. of sterile physiological saline and stored at −12°. These antigens were used at further dilutions of 1 : 10 to 1 : 500 in physiological saline.

Immunization of rats.—Various immunization schedules were employed. In all cases, rats received injections of 0·25 ml. of diluted antigen into each foot-pad. In some experiments, mixtures of 2 or 3 antigens were injected. In others, injection of one antigen was followed after some time by the injection of another antigen. Finally in some experiments, the antigen was incorporated into adjuvants, using Flozene 50 (H. C. Sleigh & Co.) as the mineral oil and Arlacel 80 (Purr-Pull Co.) as the emulsifying agent. Animals were killed 5–8 days after their last injection of antigen.

Preparation of cell suspensions and micromanipulation.—The techniques used for the preparation of single cell suspensions from the popliteal lymph nodes draining the injection site, and for the isolation of single cells in microdroplets closely followed the techniques previously described (Nossal, 1959*b*). The only change made was to select for study only those

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cells believed from their appearance at 100-fold magnification (dark-ground) to be plasma cells.

Estimation of antibody content of microdroplets.—The oil chamber carrying the single-cell microdroplets was incubated at 37° for 3½ hr. Then it was replaced on the microscope stage, and about 10 motile bacteria of one of the 2 or 3 immunizing strains were instilled into each microdroplet. If total loss of motility was observed at any time in the next 15–20 min. a further 10 bacteria of the same strain were instilled, and if they too were immobilized, a further 10 bacteria were instilled, and so on until the antibody content of the droplet had been exhausted or 5 lots of bacteria had been immobilized. The antibody titre of the droplet against this strain was then recorded as 0, 1, 1½, 2 etc. up to " 5 or greater ". For this purpose, the figure ½ meant partial immobilization of the final lot of bacteria added. The final lot was considered to be partially immobilized if 1–3 bacteria remained motile.

When the antibody titre of each droplet to the first immunizing strain had been determined, a small quantity of a 1 : 100 dilution of a specific antiserum to that strain was added to each droplet. This immobilized all the bacteria in all the droplets, providing a background against which the immobilization of further bacteria of the second immunizing strain could readily be tested. Then 10 bacteria of the second immunizing strain were added to each droplet and observed for immobilization. If immobilization occurred, the antibody titre of the droplet to that strain was determined as before. If 3 antigens had been used, the bacteria of the second strain were immobilized by a small quantity of specific antiserum as before, and the antibody titre against the third strain determined.

Staining of single cells.—In some experiments cells were stained with orcein and light green as previously described (Nossal, 1959*b*).

Serum antibody titrations.—The dilution of serum giving 90 per cent immobilization of a standard bacterial suspension was determined as previously described (Nossal, 1959*c*).

RESULTS

Assessment of antibody titration method

In early work on antibody production in doubly immunized animals (Nossal, 1958) only those cells capable of immobilizing the first strain were tested against the second strain. The amount of antibody formed against either strain was not determined. In the present studies, a new technique has been used to determine the antibody titre of all microdroplets against each of 2 or 3 strains of Salmonella. As outlined above, this involved instilling into the droplets a small quantity of specific antiserum to provide a background against which the immobilization of further, serologically distinct but morphologically indistinguishable, bacteria could be assessed. It was obviously important therefore to determine that the introduction of an excess of one antibody did not affect the titre of a second antibody present in the droplet.

Accordingly 2 immune sera were prepared against each of the 4 antigens by hyperimmunizing rats. Each serum was titrated against each strain of bacteria. These sera had titres of the order of 100,000 against the immunizing strain, but titres in the range $<$10–60 against the other strains. This indicated a very slight serological overlap between the strains. Next, microdroplets of the size usually used in single cell experiments were prepared containing the following dilutions of anti-AD serum in 30 per cent rat serum—Earle's saline : 1 : 100,000 ; 1 : 10,000 ; 1 : 5000 ; 1 : 2000 and 1 : 1000. Six droplets of each dilution were prepared, and to half of them, a small quantity of 1 : 200 anti-685 serum was added by micromanipulation. The titre of each droplet against AD was then determined. The results (Table I) showed that the presence of the anti-685 serum did not materially affect the anti-AD titre ; and that over the titre range 1–10, the anti-AD titre was approximately proportional to the dilution of immune serum.

TABLE I.—*Anti*-AD *Titres of Microdroplets of Standard Size Containing Diluted Anti*-685 *Serum*

Dilution of serum	Mean titre of droplets without anti-685	Mean titre of droplets with anti-685
1 : 100,000	<1	<1
1 : 10,000	1	1
1 : 5000	2·3	2
1 : 2000	4	4·7
1 : 1000	10·3	12.3
1 : 100	>20	>20

Furthermore, in the experiments to be reported each cell-containing droplet had beside it a control droplet containing only suspending medium and a small amount of washing fluid. The addition of antiserum to these droplets never affected the motility of bacteria of the second strain subsequently added to them.

Simultaneous double immunization with AD *and* 685.

Seventeen experiments were performed using mixtures of AD and 685 antigens. In these experiments the interval between primary and secondary immunization was 4–6 weeks and the animals were killed 5–8 days after secondary immunization. The relative doses of the 2 antigens varied widely (Table II).

TABLE II.—*Antibody Production by Single Cells from Rats Immunized with* AD *and* 685.

Expt.	Dilution AD antigen: 1ry. Immunization	Dilution AD antigen: 2ry. Immunization	Dilution 685 antigen: 1ry. Immunization	Dilution 685 antigen: 2ry. Immunization	Number of cells tested	Number of cells immobilizing AD	Number of cells immobilizing 685
1	1 : 100	1 : 100	1 : 100	1 : 100	30	13	2
2					40	19	1
3					22	5	1
4					33	10	0
5	1 : 100	1 : 100	1 : 100	1 : 10	39	5	2
6					32	12	1
7	1 : 100	1 : 100	1 : 25	1 : 100	18	0	3
8					27	5	1
9					37	5	1
10					32	9	1
11					21	2	0
12					36	2	1
13					29	2	0
14	1 : 50	1 : 500	1 : 50	1 : 50	24	3	4
15					30	1	2
16					36	1	2
17	1 : 100	1 : 100	1 : 10	1 : 10	33	9	0
Total					519	103	22

No cell produced antibody against both strains.

Of 519 cells examined, 125 produced antiflagellar antibody, 103 producing anti-AD and 22 anti-685, but no cell producing both antiflagellar antibodies. The titres produced by the single cells (Fig. 1) were well above threshold in most cases.

Simultaneous double immunization with other combinations

Six experiments were performed on rats immunized with mixtures of other combinations; 2 with 685 and 997, 2 with AD and 997 and 2 with AD and 940. In other respects, these experiments were similar to the previous series. Of 210

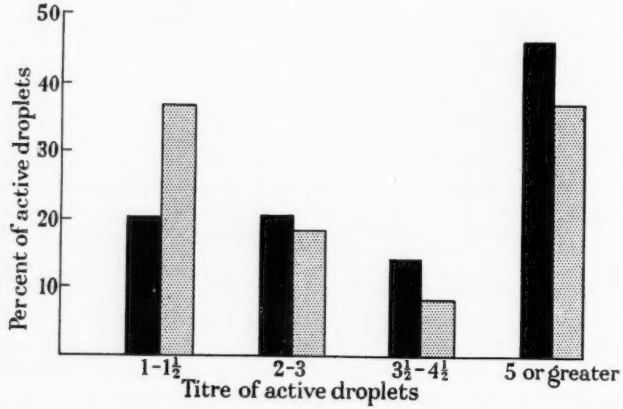

FIG. 1.—Titres of active single cell droplets from rats immunized with AD and 685.

Titres of droplets active against AD.

Titres of droplets active against 685.

cells examined, 53 produced antiflagellar antibody (Table III). No cell produced more than one antibody.

TABLE III.—*Antibody Production by Single Cells from Doubly Immunized Animals*

Expt.	Antigens	Number of cells tested	Number of cells active against	
			685	997
18 and 19	685 and 997	71	5	11
			AD	997
20 and 21	AD and 997	78	10	8
			AD	940
22 and 23	AD and 940	61	13	6
Total		210	53	

No cell produced antibody against both strains.

Simultaneous triple immunization

Ten experiments were performed in which the rats had been triply immunized with mixtures of AD, 685 and 997 antigens. In other respects, these experiments were similar to the previous series. All cells were tested against all three strains, and of 351 cells examined 104 produced detectable antibody (Table IV); 50 against

AD, 29 against 685 and 25 against 997. No cell produced antibody against 2 or all 3 strains.

The antibody titres produced by these cells are given in Fig. 2.

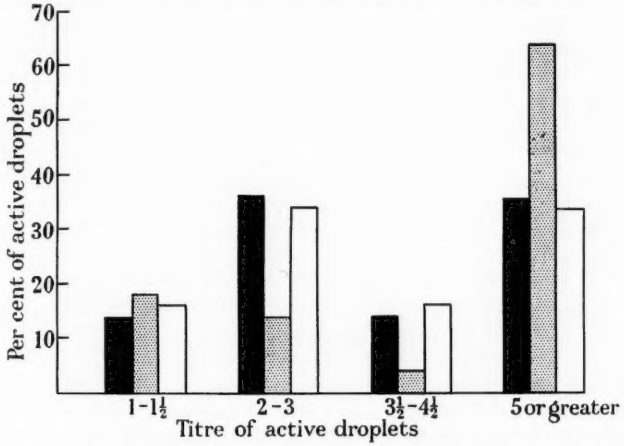

FIG. 2.—Titres of active single cells from rats immunized with AD, 685 and 997.

Titres of droplets active against AD.

Titres of droplets active against 685.

Titres of droplets active against 997.

TABLE IV.—*Antibody Production by Single Cells from Rats Immunized with Mixtures of* AD, 685 *and* 997 *Antigens*

Expt.	Number of cells tested	Number of cells immobilizing AD	Number of cells immobilizing 685	Number of cells immobilizing 997
24	36	7	5	3
25	33	7	4	3
26	43	10	5	3
27	29	4	5	3
28	31	1	0	0
29	36	2	1	5
30	30	1	3	2
31	35	4	1	3
32	36	9	1	2
33	42	5	4	1
Total	351	50	29	25

No cell produced antibody against more than 1 strain.

Sequential immunization

Five experiments were performed in which the rats had been immunized with the flagellar antigen incorporated in water-oil emulsion to give an adjuvant effect.

In one of these experiments (Expt. 34) injection of 685 in adjuvants was followed after 7 weeks by injection of AD in adjuvants, and the rat was killed 11 days later.

In 4 other experiments (Expt. 45–48), injection of 685 in adjuvants was followed after 7 weeks by injection of AD in adjuvants, and 2 weeks later by injection of 685 in saline. In all these experiments, the popliteal lymph nodes were greatly enlarged, being at least 10 times normal size. The results (Table V) show that out of 165 cells tested, 30 produced antibody ; 9 against AD and 21 against 685. No cell produced antibody against both strains.

TABLE V.—*Antibody Production by Single Cells from Rats Serially Immunized with* AD *and* 685 *in Adjuvants*

Expt.	Number of cells tested	Number of cells immobilizing AD	Number of cells immobilizing 685
34	27	4	0
35	35	2	2
36	32	1	5
37	36	1	6
38	35	1	8
Total	165	9	21

No cell produced antibody against both strains.

Five further experiments were performed on sequentially immunized rats. These received 2 pairs of injections of 940 in saline 4 weeks apart. They were injected with AD 2 days after the second injection and 4–6 weeks later, with a mixture of AD and 940. They were killed 5–6 days later. The results (Table VI) show that out of 154 cells tested, 35 produced antibody ; 27 against AD and 8 against 940. No cell produced antibody against both strains.

TABLE VI.—*Antibody Production by Single Cells from Rats Serially Immunized with* 940 *and* AD

Expt.	Number of cells tested	Number of cells immobilizing 940	Number of cells immobilizing AD
39	33	1	8
40	32	4	5
41	33	1	4
42	28	0	6
43	28	2	4
Total	154	8	27

No cell produced antibody against both strains.

Staining of cells

In about every third experiment, single cells were stained with orcein and light green. Of 337 identifiable cells, 291 (86 per cent) were plasma cells, and 17 (5 per cent) were plasmablasts. The remaining cells were medium and large lymphocytes (blasts) or polymorphs. Lymphocytes and polymorphs did not produce detectable antibody.

DISCUSSION

Since our previous report on antibody production by single cells from doubly immunized animals (Nossal, 1958), we have had much more experience of the various technical problems of single cell work, and have come to realize that the early experiments had the following serious limitations. Firstly, the amount of

antibody produced by the single cells was small. This was probably due to two reasons, namely the small size of the nutrient droplets and the early stage (3 days) after tertiary immunization at which the rats were killed. It is probable that the active cells at this stage were plasmablasts, which are not as active antibody-producers as plasma cells (Nossal, 1959*b*). Secondly, droplets may have contained small amounts of antibody prior to incubation because of inadequate washing of cells. Thirdly, comparatively few antibody-producing cells were tested. Finally, no attempt was made to test each droplet for antibody against each strain ; half the cells were tested against one strain and the other half against the other. Only droplets active against the first strain instilled were tested for antibody against the other strain.

In the present experiments, these difficulties have been overcome. Firstly, by the use of large microdroplets, and by concentrating study on more mature plasma cells taken 5–8 days after secondary immunization, it was found that most of the active cells produced amounts of antibody well in excess of the threshold (Figs. 1 and 2). Secondly, as each single cell was washed individually, the droplets before incubation were free of pre-formed antibody. Thirdly, by selecting for study only cells believed to be plasma cells we have been able to study far more antibody-producing cells. In all, 347 single-producers but no double-producers have been found. Finally, using the new antibody-titration method, the antibody content of each droplet against each bacterial strain could be assessed with reasonable accuracy (Table I, Figs. 2 and 3).

Although previous experience leads us to believe that 80–90 per cent of the cells selected in the present experiments were plasma cells (Nossal, 1959*b*), by no means all the cells produced antiflagellar antibody. Moreover, in most experiments, one antigen was more effective than the other (*e.g.* Table II). These results are the subject of further study, and will be discussed in the next paper of this series.

The present experiments confirm the observation that each antibody-producing cell can form only one antibody at one time. It is shown that out of 729 cells examined from animals immunized with mixtures of 2 antigens, 178 formed one or other antiflagellar antibody but none formed both (Tables II and III). Failure to find a double-producer does not mean that these do not occur. If the 729 cells are considered as derived from one population, failure to find a double-producer means that the incidence of double-producers in that population lies between 0 and 0·004 (E. S. Pearson and H. O. Hartley's Tables for 95 per cent confidence limits). We have studied a further 351 cells from animals immunized with 3 antigens, of which 104 formed one or other antibody. No double or triple-producers were found.

Other workers have approached this problem by the fluorescent antibody technique (Coons, 1958 ; White, 1958), which is capable of testing a large number of cells for the content of one or both of two antibodies. So far, no double-producers have been reported.

It has been suggested (Lederberg, 1959) that serial immunization of an animal first with one antigen and then with another could favour the multiplication of cells capable of forming both antibodies. To test this hypothesis, rats were immunized serially in 10 experiments. In the first 5 (Table V), rats received injections of the first antigen in adjuvants and 7 weeks later of the second antigen also in adjuvants. In the next 5 (Table VI), rats were given 2 injections of the first

antigen in saline 4 weeks apart, and then 2 days later, when extensive multiplication of antibody-forming cells would have been just beginning (Nossal, 1959*b*; Leduc, Coons and Connolly, 1955) the second antigen was injected. In 9 of these 10 experiments, the animals received a further injection before killing. No double-producers were found.

Following the injection of large amounts of 2 or 3 antigens into the foot-pad, it is likely that most cells in the draining popliteal lymph nodes encounter many molecules of both or all three antigens. There seem to be two main alternative explanations for the failure to find double or triple antibody producers. Firstly, it is possible that once a cell initiates antibody production corresponding to a particular antigenic determinant, the protein synthetic mechanism is pre-empted for that task, and incapable of producing a second antibody. Secondly, the secondary response might result from a clonal multiplication of specifically pre-adapted cells. Double immunization might lead to the proliferation of 2 distinct clones, one pre-adapted to form one antibody, the other to form the second. If this view were correct, it still remains to be determined whether the cells were pre-adapted *ab initio*, as in Burnet's (1957, 1959) clonal selection hypothesis, or whether they are the descendants of cells genetically modified by contact with antigen during the primary response.

SUMMARY

Further work on the phenotypes of antibody producing cells from animals immunized with 2 or 3 antigens is reported. Using a modification of previously described techniques for detecting antibody-production by single cells, cells from rats immunized with mixtures of 2 Salmonella flagellar antigens were tested for antibody production. Of these, 125 produced one or other antibody but none produced both. A further 351 cells from rats immunized with 3 antigens contained 104 single producers, but no double or triple producers. When rats were serially immunized with 2 antigens, similar phenotypic restriction of antibody-producing capacity was observed amongst 154 cells removed some weeks later.

I wish to thank Sir Macfarlane Burnet for his stimulating interest and help; Professor Joshua Lederberg for many suggestions; and Miss Wendy McDonald for excellent technical assistance.

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SURVEY OF PAPERS

GILLMAN, GRANT and HATHORN report the results of a combined chemical, histological and histochemical study on the tissues following calciferol-induced injuries in rats. Rats were fed with calciferol in arachis oil in varying doses, subsequently killed and fixed *in toto*. The heart, aorta, kidney, stomach, bowel, spleen and lymph nodes were subsequently removed. These tissues were subjected to analysis with special reference to the hexose, hexosamine, collagen, elastin, calcium and phosphorus contents of the aorta, heart and mucoprotein and calcium levels of the serum.

Calciferol-induced injuries resulted in mineralization of the aorta which persisted by contrast to the absence of increased serum calcium and the absence of mineral in the heart and coronary vessels. The increase in aortic calcium was closely paralleled by the increase in the phosphorus content, and there was also an elevation of the serum mucoprotein. The hexose and hexosamine levels in the aorta were also increased and, with developing aortic sclerosis the amount of collagen was present in excess. These findings are discussed in relation to the pathogenesis of atherosclerosis (p. 1).

GINSBURG, LAUFER and ROSENBERG produced muscle necrosis and granulomatous lesions in the myocardium of rabbits by the intra-cardiac injection of various organisms.

The association of trauma to the heart muscle and haemolytic streptococci or lactobacilli, *Bact. coli* and non-haemolytic streptococci produced identical histological lesions in the myocardium.

Trauma to the heart alone or intravenous injection of these organisms failed to produce myocardial lesions (p. 19).

JENKIN and ROWLEY have continued their work on experimental cholera in rabbits. They show that animals immunized against *V. cholerae* are resistant to the experimental infection. Also they demonstrated resistance to the experimental infection in weanling rabbits that had *V. cholerae* presensitized with antibody injected into the small intestine (p. 24).

HARNDEN investigated the human chromosome complement in 9 pieces of skin and one portion of testis by a tissue culture technique.

The tissue culture method described has proved very successful, all 10 resulted in an actively growing culture. By this technique the author has confirmed the deviations in the chromosome numbers in cases of Klinefelter's Syndrome, Turner's Syndrome and in a patient with both Klinefelter's Syndrome and mongolism (p. 31).

BURROWS and BACON have examined strains of *Pasteurella pseudotuberculosis* for the presence of V and W antigens. They found that the majority of recently isolated strains were VW+, that these VW+ strains usually consisted of mixed population of VW+ and VW— organisms and that repeated subculture resulted in the elimination of the VW+ organisms (p. 38).

GRANT, continuing his study of the development of turbidity and precipitation in antigen-antibody mixtures, has used pneumococcal polysaccharide antigens. He found that dioxan treatment of the mixture or the antiserum (but not the polysaccharide) did not affect turbidity but delayed or inhibited precipitation. While ethanol intensified turbidity without inhibiting precipitation. These results differed from the author's earlier findings in protein antigen systems, where dioxan inhibited turbidity. In an extensive discussion explanations for these differences are suggested (p. 45).

MIMS has studied the pressures and volumes usually employed in intracerebral injection in mice ; and has found, by the fluorescent antibody technique, the sites of multiplication of some viruses after introduction by this route. Ectromelia multiplied in cells of the meninges and ependyma and Murray Valley encephalitis in neurones and neuroglial cells (p. 52).

GARDNER observed focal segmental necrotizing arteritis and widespread focal arteriolitis in rats treated with salt and cortexone. When animals were simultaneously treated with hydralazine once daily, neither form of vascular lesion developed. The daily injections of hydralazine caused severe repetitive fluctuation in the blood pressure, each dose being followed by a profound fall in pressure which was succeeded by a slow rise to the preinjection level. He deduces from these results that a critical rise in blood pressure is not an adequate explanation for the appearance of acute arterial and arteriolar lesions in experimental hypertension (p. 60).

PORTER investigated male Chinchilla rabbits irradiated and injected with twenty day foetal rabbit liver and adult marrow intravenously. Red cell survival was estimated using ^{51}Cr labelled cells.

In rabbit radiation-chimaeras which develop " secondary disease " there is rapid destruction of ^{51}Cr labelled host cells and this haemolytic destruction is selective in that the labelled donor cells are unharmed. The accompanying positive Coomb's test suggests that the haemolysis is an immune response and the syndrome of the " secondary disease " is regarded as an immune reaction by the graft against the host. The jaundice which may appear only occurs in those ailing rabbits with cirrhosis of the liver due to coccidiosis (p. 72).

ROBSON and SMITH vaccinated mice with *Myco. lepraemurium* or *Myco. tuberculosis* (BCG) and challenged them with intracorneal *Myco. lepraemurium* after 12 or 74 days. They found that the course of the corneal infection was unchanged in the animals challenged after 12 days, but that multiplication of the organisms was less in those challenged after 74 days regardless which organism had been used for vaccination (p. 81).

DALE, with a micrometer syringe, made subcutaneous injections in the right ears of mice. Of 3 groups of mice, one received saline, a second turpentine and the last an adjuvant emulsion. Four weeks later, both left and right ears were examined, the lymphatics demonstrated by injection with Indian ink, and photographs made. Saline caused no changes, turpentine caused macroscopic damage and some lymphatic proliferation and the adjuvants produced this proliferation without damage (p. 86).

NOSSAL has isolated, in microdroplets, single lymph node cells from rats immunized against more than one bacterial antigen. He selected those which appeared to be plasma cells and the success of selection was checked by staining some of them. Production of antibody by these cells was detected and measured by the introduction of successive batches of motile bacteria into the droplets until no further immobilization occurred. Antibodies were produced against all the antigens received by the animal, but no cell produced more than one antibody. These results are not inconsistent with the " Clonal Selection " theory of acquired immunity (p. 89).